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DIPLOMA THESIS

TRAIL-induced Apoptosis in Populations of Colon Cancer Cell Lines under Various Cultivation Conditions

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I hereby declare that this diploma thesis is my own work and effort. I have referenced all literature sources and I have worked under supervision of RNDr. Ladislav Anděra, CSc.

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Abstract

Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) is a cytokine of TNF family, which participates in the non-exclusive regulation of survival and proliferation of mainly hematopoietic cells. Shortly after its discovery it also brought significant attention as specific and potent inducer of apoptosis of cancer cells of various origins, and since then it has been investigated as a potential novel anti-tumor therapeutics. Recently, cancer stem cells (CSCs) were suggested to be a distinct subset of tumor cells that could be responsible at least in some tumors for their sustainment, recurrence and drug resistance. These cells in the "hierarchic" model of tumorigenesis thus represent an important and attractive target for efficient tumor therapy.

In this study we use several colorectal adenocarcinoma cell lines as an experimental model for the analysis of CSC-prone cultivation conditions on TRAIL-induced apoptosis of these cells. For enrichment of eventual cancer stem cells we cultivated cell lines in a serum-free medium, originally developed for cultivation of neural stem cells, and assessed the expression of putative CSC markers CD133 and ABCG2 by flow cytometry (FACS). Simultaneously, we tested the expression of TRAIL receptors and susceptibility to TRAIL-induced apoptosis in these cells. We observed correlation between appearance of stem cell-like signs (spheroid formation, expression of CD133) and increased resistance of these cells to TRAIL-induced apoptosis. Profiling of surface proteins revealed that compromised apoptosis was not inherent in expression of death receptors, since these were mostly upregulated after serum-free cultivation.

Recently it has been reported, that fibroblasts isolated from tumor stroma could induce de-differenitated, stem cells-like phenotype in normal human keratinocytes and thus these stromal cells represented an attractive tool for the analysis of their effect on TRAIL-induced apoptosis of co-cultivated tumor e.g. colon cancer cells. In our initial attempt we analyzed effect of both normal skin and tumor stroma-derived (basal cell carcinoma, spinal cell carcinoma and benign fibrous histiocytoma) fibroblasts on TRAIL-induced apoptotic signaling in selected colorectal adenocarcinoma cell lines. We observed no unequivocal effect of either stromal or normal fibroblasts on TRAIL-induced apoptotic signaling in two co-cultivated colon cancer cell lines HCT 116 and DLD-1. We cannot however exclude possible cancer cell-type specific influence of stromal cells on TRAIL-induced apoptosis and thus further analysis using different cancer cell lines is required.

Key words

apoptosis, cytokines, TRAIL, death receptors, tumor, chemotherapy, cancer stem cells, niche, spheroid bodies, stromal fibroblasts

List of abbreviations

aa	amino acid
ABB	annexin V-binding buffer
ABCG2	ATP-binding cassette G2
AIF	apoptosis inducing factor
Apaf-1	apoptosis protease activating factor-1
Apo-2	death receptor 4/TRAIL receptor 1
ATP	adenosine 5'-triphosphate
B cell	B lymhocyte
Bad,	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated X protein
Bcc	basal cell carcinoma
Bcl-2	apoptosis regulatory protein discovered in B-cell lymphoma
Bcl-x_L	apoptosis regulatory protein allied to Bcl-2
Bfh	benign fibrous histiocytoma
BH	Bcl-2 homology domain
Bid	BH3-interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-like 11 protein
Bmf	Bcl-2-modifying factor
BMP	bone morphogenetic protein
Bok	Bcl-2-related ovarian killer
CD	cluster of differentiation
CMTPX	CellTracker Red dye
CSC	cancer stem cell
C-terminus	carboxy-terminus/COOH-terminus
CAF	cancer-associated fibroblast
CARD	caspase recruitment domain
CRD	cysteine-rich domain
Cys	cysteine
CXCR4	receptor of SDF-1
cyt c	cytochrome c
Da; kDa	Dalton (molecular mass unit 1Da ≈ 1.6605×10^{-27}kg); kiloDalton
DC	dendritic cell
DcR	decoy receptor
DD	death domain
DED	death effector domain
DIABLO	direct IAP-binding protein with low pI/murine homolog of Smac
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DP5	see Hrk
DR	death receptor

ECM	extracellular matrix
e.g.	for example
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
etc.	*et cetera*
FADD	Fas-associated death domain (adaptor protein)
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FBS	fetal bovine serum
bFGF	basic fibroblast growth factor
FITC	fluorescein isothiocyanate
FLICE	FADD homologous ICE-like protease/caspase-8
FLIP	FLICE-like inhibitory protein
GPI	glycosylphosphatidylinositol
His	histidine
Hrk	hara-kiri/Bcl-2-interacting protein/DP 5
i.a.	*inter alia*
IAP	inhibitor of apoptosis protein
ICE	caspase-1
IFN	interferon
IMDM	Iscove's Modified Dulbecco's Media
JNK	Jun N-terminal kinase
mAb	monoclonal antibody
MAP	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia protein-1
mDR-5	murine death receptor-5
MOMP	mitochondrial outer membrane permeabilization
mRNA	messenger ribonucleic acid
mTRAIL	murine Tumor-necrosis factor-Related Apoptosis Inducing Ligand
N-terminus	amino-terminus/NH2-terminus
NF-κB	nuclear factor κB
NK; NKT	natural killer cell; natural killer T cell
Noxa	BH3-only member of the Bcl-2 family
NS-Ac	serum-free medium (complete)
OPG	osteoprotegerin
p53	transformation-related protein 53
PBS	phosphate-buffered saline
PCD	programmed cell death
PE	phycoerythrin
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
Puma	p53-upregulated modulator of apoptosis
RANK	receptor activator of NFκB
RANKL	receptor activator of NFκB ligand

RFP	red fluorescent protein
RIP	receptor-interacting protein
RLU	relative luminescence unit
ROS	reactive oxygen species
RPMI	cultivation medium (RPMI 1640)
Scc	spinal cell carcinoma
SDF-1	stromal cell-derived factor 1
siRNA	small interfering ribonucleic acid
Smac	second mitochondria-derived activator of caspase
SRP	signal recognition particle
T cell	T lymphocyte
TIC	tumor initiating cell
tBid	truncated form of BH3-interacting domain death agonist
T_H1	helper T lymphocyte type 1
T_H2	helper T lymphocyte type 2
THD	TNF-homology domain
TNF-α	Tumor-necrosis factor-α
TNFR	Tumor-necrosis factor receptor
TNFSF 10	Tumor-necrosis factor superfamily member 10
TRADD	Tumor-necrosis factor receptor-associated death domain protein
TRAIL	Tumor-necrosis factor-Related Apoptosis Inducing Ligand
TRAIL-R	Tumor-necrosis factor-Related Apoptosis Inducing Ligand receptor
WIPO	World Intellectual Property Organisation
XIAP	X-linked inhibitor of apoptosis protein
σ	standard deviation

Contents

I. Introduction

Metazoan ontogeny represents a sequence of tightly regulated processes leading to a stable architecture of the adult body. In case of sexually reproducing organisms, this sequence starts at zygote, which further divides in a well organized manner and constitutes primitive cells of the embryo. As these cells further perpetuate themselves and generate differentiated progeny, levels of complexity are being added eventually leading to highly complex functional tissues and organs of the newly arising individual. Most likely it is cellular hierarchy and polarization through which the information of a body plan is ultimately being executed at all aforementioned levels of development (Wodarz, 2002). Not only this idea is intuitive, it has been supported by mathematical modeling that hierarchy applies as a background principal essential for the maintenance of tissue homeostasis, suggesting its importance for both developing and adult organism (Paulus et al., 1992). A crucial question remains as to how the hierarchy is initiated and namely whether its constitution is a onetime or recurrent process during the lifespan of an individual.

By contrast, it is probable that cancer is a group of diseases emerging directly from abusing certain developmental principles. Despite of many hollow places in our basic understanding of cancerogenesis, its ultimate lethality is based on unregulated cell growth and crippled tissue homeostasis. In other words, the mechanisms responsible for maintenance of cell counts must be unbalanced. One such mechanism, apoptosis, has been in focus of cancer research since its discovery more than century ago. This form of programmed cell death is an evolutionary conserved process that is in multicellular metazoans and plants required for elimination of no-longer needed or dangerous (e.g., infected or malignant) cells (Joza et al., 2002). Unlike necrosis, apoptosis relies on firmly regulated cascades of finely-tuned events that finally lead to organized destruction of the dying cell, relatively gentle to the surrounding tissue (Saraste and Pulkki, 2000).

Combining the rapidly growing developmental perception of cancer that has recently concentrated around the so called “cancer stem cell” (CSC) hypothesis with the fact that apoptosis is strongly involved also in fundamental developmental processes, it is a tempting idea to search for signals that could eventually initiate apoptosis in hierarchically outstanding cells.

Several lines of evidence have recently hinted that Tumor-necrosis factor-Related Apoptosis Inducing Ligand (TRAIL) is a promising molecule for selective killing of cancerous cells. For possible consequences of TRAIL with anti-tumor/anti-metastatic immune surveillance (Grosse-Wilde et al., 2008), we decided to investigate the sensitivity of differentially cultured colon cancer cells to TRAIL-induced apoptosis.

Following aims of the study were set:

1. To characterize given colon cancer cell lines (morphology, sensitivity to TRAIL-induced apoptosis, expression of surface markers) depending on various cultivation conditions

2. To asses phenotype of colon cancer cell lines in relation to cancer stem cell hypothesis

3. To develop co-cultivation design for investigation of direct influence of tumor stromal fibroblasts on cancer cell phenotype

4. To assess the potential of TRAIL as a anti-CSC agent

II. Review of the literature

II.1 TNF-Related Apoptosis Inducing Ligand (TRAIL) and its role in tumorigenesis

TRAIL or Apo2 ligand (TRAIL/Apo2L/TNFSF10) is a cytokine of the TNF superfamily, whose members share a TNF structural motif – TNF Homology Domain (THD). At present the family consists of 19 members that activate diverse cellular processes as proliferation, differentiation and apoptosis (Aggarwal, 2003). Their cognate receptors, TNF receptor (TNFR) superfamily, consists of 29 members and are distinguished by presence of cysteine bridges-containing pseudorepeats, also called cysteine rich domain (CRD) in their extracellular parts. A subgroup of the receptors within TNFR superfamily contains a death domain (DD) and can upon activation induce apoptosis of the receptor expressing cells (Aggarwal, 2003).

TRAIL was discovered in 1995 by THD homology-based computer search in expressed a sequence tag (EST) library (Wiley et al., 1995). For its specific tumor cells-induced apoptosis, TRAIL received significant attention as potentially novel anti-tumor agent. In contrast to other members of TNF family (e.g. TNF-α or FasL), systematic administration of the recombinant TRAIL did not lead to any toxic side effects neither in mice nor in primates (Lawrence et al., 2001). TRAIL is thus being a rare example of such molecules that can eliminate many transformed cells while sparing normal ones. Moreover, TRAIL can induce tumor cells apoptosis independently of their p53 status (Nagane et al., 2001) (tumors with mutated p53 are more difficult to eradicate using the conventional chemotherapy). This offers an attractive possibility of simultaneous administration of TRAIL and widely-used anti-cancer drugs or radiation treatment. Synergistic effects of TRAIL with etoposide, cisplatin, irinotecan oxaliplatin; protease inhibitors MG321 and bortezomib and γ-irradiation on enhancing tumor cells apoptosis have been documented in a number of reports (reviewed in (Duiker et al., 2006)) . At the present are ongoing phase I/II clinical trials with both recombinant TRAIL (Genentech)

and with the agonist humanized anti-TRAIL-R1 and -R2 monoclonal antibodies (Human Genome Sciences) and the up to now results look very promising (Duiker et al., 2006).

II.1.1 Structural and biochemical characterization of TRAIL

Human gene for TRAIL, is localized at 3q26 of chromosome 3 and encodes a 281 aa protein with predicted molecular weight 32509 Da (Wiley et al., 1995). TRAIL is a typical type II transmembrane protein, with one transmembrane hydrophobic region and absent signal sequence. Although TRAIL occurs mostly in membrane-bound form, it can be also proteolytically cleaved, albeit apoptosis-inducing activity of this soluble form is compromised (Mariani and Krammer, 1998).

As other members of the TNF superfamily, TRAIL contains THD and forms "bell-shaped" trimers (**fig. II. 1**). Common features of THDs are a very similar tertiary fold and their ability to form trimeric proteins (Mariani and Krammer, 1998). Crystallographic studies revealed that each subunit contains a rigid frame of two anti-parallel beta sheets (beta sheet sandwich structure) and loops connecting individual sheets are highly disordered in the thinner part of the bell. TRAIL is unique within the TNF superfamily as it contains a 15 residue-long extension in one loop that spans the complete outer surface of the monomer (Cha et al., 2000). This elongated loop is suggested to enable specific recognition of TRAIL receptors (Cha et al., 2000). Another exceptional feature of the TRAIL is presence of zinc ion-binding site made of cysteine (Cys)-230 residues at the trimer interface, which is crucial for the maintenance of optimal biological activity (Hymowitz et al., 2000).

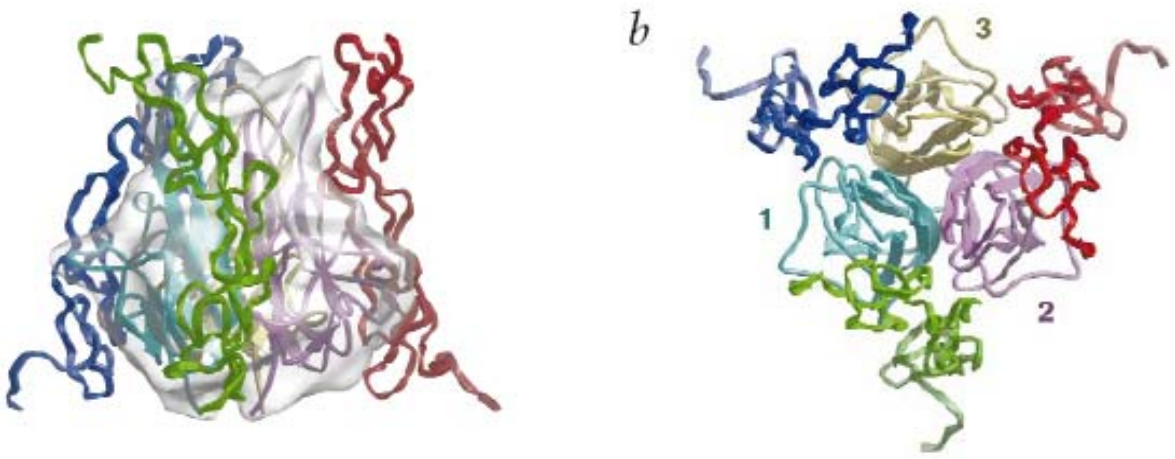


Figure II. 1: The structure of the TRAIL-DR5 complex. Left: trimer of TRAIL subunits (yellow, cyan, pink) and DR5 (blue, green, red); TRAIL trimer is enclosed in a transparent molecular envelope. Right: the complex viewed down the three fold axis; ad (Mongkolsapaya et al., 1999).

II.1.2 TRAIL Receptors

TRAIL interacts with five trimeric receptors, whose discovery opened yet not resolved discussion about a purpose of their multiplicity. Only two of the five TRAIL receptors, namely death receptor 4 (DR4/TRAIL-R1) and death receptor 5 (DR5/TRAIL-R2) are capable of inducing cell death after TRAIL ligation. This is thanks to death domains (DD) in their intracellular portions. DD is a specific protein-interaction domain which serves as a binding site for essential pro-apoptotic adaptor proteins. DR5 and shares 58 % homology with DR4 and both receptors are expressed in wide range of tissues and are strongly upregulated on activated lymphocytes (MacFarlane et al., 1997).

As TRAIL signaling is proposed to play an important role in tumor immune surveillance, mutations in components of TRAIL signaling pathway can occur during tumorigenesis. Polymorphism in the ligand-binding domain of DR4 has been connected with a higher risk of bladder cancer, and other mutations within DR4 have been reported in cancer cells (Hazra et al., 2003). Conversely, the expression of non-mutant DR4 in colon tumors was linked to favorable prognosis (Strater et al., 2002).

Existence of two death receptors for the same ligand could indicate their distinct role in regulation of tissue homeostasis or activation of secondary signaling pathways such as ERK, JNK, p38 MAP kinase and NF-κB ones (Malhi and Gores, 2006)[1] (**fig. II. 4**.)

In support of their distinct roles, Muehlenbeck et al. (2000) using DR5-specific TRAIL mutants showed that the JNK pathway is preferentially activated by DR5. Colon cancer cells preferentially use DR5-triggered apoptosis (Wang and El-Deiry, 2004) and in contrast, chronic B cell leukemia cells activate upon TRAIL ligation DR4-dependent apoptosis (MacFarlane et al., 1997). Also their transport to the cell surface is differently regulated as knockdown of signal recognition particle (SRP) protein SRP72 compromises only DR4 but DR5 cell surface localization (Ren et al., 2004). Increased DR5 expression could be characteristic for transformed cells as majority of normal cells express only low levels of DR5 at the cell surface (Wang and El-Deiry, 2004).

Unlike death receptors, so-called decoy receptors upon ligation with TRAIL do not induce cell death. Decoy receptor 1 (DcR1/TRAIL-R3) lacks the cytoplasmic part and is attached to the membrane through a glycosylphosphatidylinositol (GPI) anchor. It is less widely expressed than other TRAIL receptors (Degli-Esposti et al., 1997). Decoy receptor 2 (DcR2/TRAIL-R4) is relatively more expressed than TRAIL-R3 and has got a high degree of homology to the other TRAIL receptors. It has transmembrane region and a truncated death domain lacking two thirds of its regular length (Marsters et al., 1997). The initial interpretation of their function was that they could serve as "decoys" competing with DR4/5 for binding to TRAIL. Nevertheless, this "decoy hypothesis" was challenged after experiments using monoclonal antibodies specific for each of the membrane-bound TRAIL receptors found no correlation between TRAIL resistance and decoy receptors expression. However, several new lines of evidence recently implied that forementioned receptors

[1] Interestingly, the proteins that are required for apoptosis and NF-κB activation are recruited competitively (Jin and El-Deiry, 2006). In TRAIL-resistant tumor cells activation of these secondary signaling pathways (mainly NF-κB) enhances their proliferation potential. Thus, TRAIL binding can promote tumor growth and survival in such cells (Malhi and Gores, 2006).

actually act as effective DR antagonists at least in some tissues, thus the "decoy hypothesis" is not absolutely refuted (Merino et al., 2006).

Third antagonistic TRAIL receptor is not membrane-bound but soluble osteoprotegerin (OPG). OPG is also high affinity decoy receptor for RANKL (receptor activator of NF-κB ligand). However, no functional relation between RANK-RANKL and TRAIL-TRAIL-R systems has been reported up to date (Vitovski et al., 2007).

II.1.3 TRAIL-induced apoptosis

II.1.3.1 Executive mechanisms of apoptosis

Signaling pathways leading to apoptosis are usually divided according to their triggers, which are either extracellular or intracellular (Fig. 2). Extrinsic pathway is directly dependant on signals from environment e.g. death ligands recognized by death receptors (Hipfner and Cohen, 2004). The main activators of the intrinsic apoptotic signaling are reactive oxygen species (ROS), misfolded proteins or damaged DNA, viral infection, and oncogenes (Kowalik et al., 1995; Lowe et al., 1994). In case of TRAIL-induced apoptosis, extrinsic and intrinsic signaling pathways may be combined via complex interactions of the molecules they involve (Jin and El-Deiry, 2006).

II.1.3.2 Caspases as apoptosis executioners

Caspases are site-specific proteases, which cleave tetrapeptide recognition motif in the target protein after aspartate residue. Caspases are expressed as inactive zymogens and require proteolytic processing and dimerization for their full activation. Active caspases contain cysteine in their active center and form heterotetramer containing two large (20 kDa) and two small (10 kDa) subunits (Cohen, 1997; Riedl and Shi, 2004).

Caspases involved in apoptosis are split into two groups. *Initiator* or "*priming*" caspases are activated in specific multiprotein complexes after recruitment via their characteristic long N-terminal prodomains containing either the death effector domain (DED) (e.g., caspase-8/-10) or the caspase recruitment domain (CARD) (e.g., caspase 9). Upon activation, the initiator caspases cleave *effector* or "*downstream*" caspases (e.g.,

caspase-3/-6/-7) or other proteins at their specific aspartate-containing sites. This processing and following conformational change-induced activation of downstream caspases then unlashes cleavage of wide spectrum of vital proteins as cytoskeletal components (actin, lamins), cell-cycle regulators, anti-apoptotic proteins, inhibitors of nucleases, components of DNA synthesis/repair machinery, kinases etc. (at present over one hundred caspase substrates are described) and subsequent dismantle of cell from within (Degterev et al., 2003).

Since caspases function as apoptosis executors, they are potentially dangerous and must be tightly regulated to prevent undesirable cell death. Caspase activity is therefore in these processes as well as during apoptosis controlled by cellular inhibitors as caspase-8 competitor FLIP or by inhibitors of apoptosis proteins (IAPs) (Riedl and Shi, 2004).

II.1.3.3 Extrinsic pathway sequence in TRAIL-induced apoptosis

II.1.3.3.1 Ligation of receptors – early events

The main signaling TRAIL receptors are TRAIL-R1/DR4 and TRAIL-R2/DR5 (Kimberley and Screaton, 2004) which represent a typical triggers of extrinsic apoptotic pathway. TRAIL death receptor signaling does not have to induce apoptosis by default, but under certain conditions when apoptosis is inhibited it also induces proliferation of the target cells (Malhi and Gores, 2006). Ligation of DR4 and DR5 by trimeric TRAIL leads to formation of intermediate activation complexes that in the apoptotic branch of their intracellular signaling contain the adapter protein FADD. FADD in addition to receptor-interacting DD also contains a death effector domain (DED), which serves for subsequent recruitment of procaspase-8 and/or -10 and assembly of Death-Inducing Signaling Complex (DISC). Proximity-activated processing of the initiator procaspases leads to their full activation and unlashing the downstream signaling that eventually activates the effector caspases and cell death (Cohen, 1997).

TRAIL receptors may be a subject of regulation and therefore may also represent first source of TRAIL-resistance. Despite the infirmed decoy hypothesis, another way of apoptosis regulation by decoy receptors was suggested. Mérino *et al.* (2006) recently proposed a model, in which TRAIL-R3/DcR1 titrates TRAIL within lipid rafts, while

TRAIL-R4/DcR2 forms inactive trimers with DR5. Receptors-associated TRAIL resistance may be also important in tumorigenesis. In metastatic breast cancers, a missense mutation G-to-A at nucleotide 422 changing histidine for arginine at codon 141 (R141H) occurs in or near the ligand-binding domain of DR4 possibly affects receptor trimerisation or ligand binding. (Shin et al., 2001).

II.1.3.3.2 Formation of DISC and downstream events

Assembly of the DISC is an essential molecular event in the signaling pathway of TRAIL-induced apoptosis and downstream apoptotic signaling divides cells into two groups (Aggarwal, 2003). In type I cells, caspase-8 is activated at the DISC in quantities large enough to directly activate effector caspases as caspase-3. In type II cells, however, the amount of active caspase-8 produced at the DISC is smaller and insufficient for effective activation of the effector caspases. In these cells a mitochondrial amplification loop is required for full activation of caspases (described in details later). Differences between type I and type II cells can have important implications for cancer cell resistance mechanisms (Aggarwal, 2003).

The fact that absence of either of DISC components was found in different types of tumor cells suggested that loss of functional DISC contributed to tumor development. Loss of caspase-8 expression has been demonstrated in malignant medulloblastomas, Ewing tumor, rhabdomyosarcomas, retinoblastomas and small cell lung carcinomas (Hopkins-Donaldson et al., 2003; Zuzak et al., 2002). In this respect, inactivating caspase-8 gene mutations have also been described in some types of tumor cells such as colorectal carcinoma and squamous carcinoma cells (Mandruzzato et al., 1997).

Competitive inhibitor of procaspase-8, FLICE-like inhibitory protein (FLIP) can, when overexpressed, protect cells from death receptor-induced apoptosis. Indeed FLIP overexpression was detected in colonic adenocarcinomas, hepatocellular carcinoma and melanoma (Ryu et al., 2001). Despite findings that overexpression of FLIP increase resistance to TRAIL, physiological function of FLIP remains unclear. It was recently suggested that FLIP may help cancer cells to acquire some degree of immune privilege (French and Tschopp, 1999).

II.1.3.4 Role of mitochondria and TRAIL-induced intrinsic apoptotic loop

Mitochondria play crucial role in apoptosis, standing on the crossroad of multiple apoptotic signaling pathways that serve as an integrator of largely intrinsic but also some extrinsic signals (**fig. II. 3**) (Joza et al., 2002).

In mitochondria reside molecules that can promote cell death in either caspase-dependent (cytochrome c) or caspase-independent manner (apoptosis-inducing factor (AIF)) (Ravagnan et al., 2002). Cytochrome c (cyt c) is soluble 12kDa protein located in intermembrane space of mitochondria serving as a part of electron transport chain. When released, it forms heptameric structure together with caspase-9, dATP and Apoptotic protease activation factor-1 (Apaf-1), so called apoptosome (Adrain and Martin, 2001). In this multiprotein complex, caspase-9 undergoes activation by proteolytic cleavage and subsequently activates processing of effector caspases and caspase-dependent apoptosis. In mitochondria reside also other pro-apoptotic molecules as IAP inhibitors Smac/DIABLO and HtrA2/Omi or nucleases AIF or endonuclease G that can apparently promote PCD without activation of downstream caspases (Ravagnan et al., 2002).

The principal regulators and promoters of apoptosis connected with mitochondria are proteins of the Bcl-2 (discovered in B-cell lymphoma) family (Cory et al., 2003). This relatively large group of signaling molecules consists of both pro- and anti-apoptotic proteins, whose common feature is presence of one to four so-called Bcl-2 homology (BH) domains (**fig. II. 2**).

Bax, Bak and Bok are members of the Bax-like subfamily of pro-apoptotic proteins and contain BH1, 2 and 3 domains. In inactive form Bax resides in cytosol but Bak is already located at the mitochondria and their localization/activity dramatically changes after an apoptotic stimulus. Bax moves to mitochondria where it similarly as Bak undergoes structural changes and via multimerization Bax/Bak produce large pores in the outer mitochondrial membrane, leading to mitochondrial outer membrane permeabilization (MOMP) and release of the pro-apoptotic proteins as cytochrome c or AIF from the intermembrane space. In contrast to Bax proteins most of the anti-apoptotic Bcl-2 proteins as Bcl-2, Bcl-x_L, Mcl-1, Bcl-W, Bcl-G contain one additional BH4 domain. Major function

of these anti-apoptotic proteins is dimerization-mediated inhibition of the pro-apoptotic, pore-forming activities of Bax proteins. Bcl-x_L moreover negatively regulates Apaf-1 and apoptosome formation. Higher Bcl-2 and Bcl-x_L level are also associated with tumor resistance to chemotherapy (Danial and Korsmeyer, 2004).

Finally, BH3-only proteins represent the third subgroup of the Bcl-2 family. These proteins contain only BH3 domain and function as sensors or sentinels of cellular stress (Huang and Strasser, 2000). In mammalian cells apoptotic signaling activated by BH3-only proteins is complex and at the present there is known over ten BH3-only proteins (e.g., Bim, Bid, Bad, Bik, Noxa, Puma, Hrk/DP5, Bmf). Individual BH3-only proteins are responsible for apoptotic responses to various kinds of cellular stress and are activated through posttranslational modification (Bim, Bad, Bmf), or *de-novo* transcription (Puma, Noxa, Hrk). Activated BH3-only sentinels then translocate to mitochondria where they bind to and inactivate anti-apoptotic, Bax-inhibitory function of the Bcl-2 proteins (Huang and Strasser, 2000).

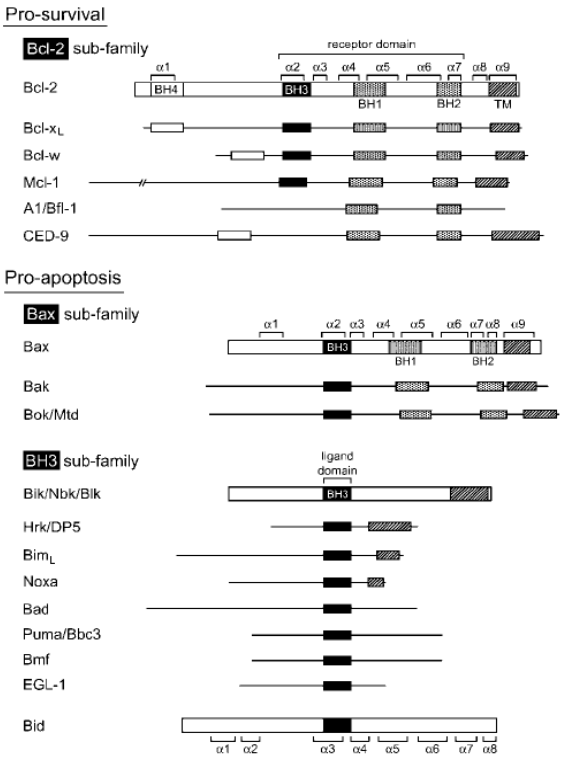


Figure II. 2: Three subfamilies of Bcl-2 related proteins; ad (Henson et al., 2003)

Mitochondrial level of TRAIL-induced apoptosis propagation is mainly important regarding type II cells. As mentioned above these cells need mitochondrial amplification

loop for successful propagation of cell death (Danial and Korsmeyer, 2004). This is achieved by cleavage of a Bcl-2 family member Bid. When cleaved by caspase-8, emergent tBid (truncated Bid) translocates to the mitochondria, where it together with Bax/Bak participates in activation of the mitochondrial apoptotic signaling Bcl-2 protein family and mitochondrial inhibitor of IAPs named Smac/DIABLO play the main roles at this level of regulation (**fig. II. 3**).

Retroviral expression of Bcl-x_L in originally TRAIL-sensitive cell line Colo357 rendered these cells TRAIL-resistant (Strater et al., 2002). Interestingly Bcl-x_L-overexpressing Colo357 cells had the same degree of caspase-8 cleavage as the original TRAIL-sensitive cells suggesting that caspase-8 activation was upstream of the mitochondrial pathway. In another study overexpression of Bcl-2 conferred protection against TRAIL in neuroblastoma and glioma cell lines (Fulda et al., 2002). In this case TRAIL-induced caspase-8 cleavage was reduced suggesting that caspase-8 was activated both upstream and downstream of mitochondria in these cells. As apoptosis induced by chemotherapy acts mainly through the mitochondrial pathway (Danial and Korsmeyer, 2004), downregulation of. Bcl-2 and Bcl-x_L might restore sensitivity not only to chemotherapy but also to TRAIL in these types of cancer.

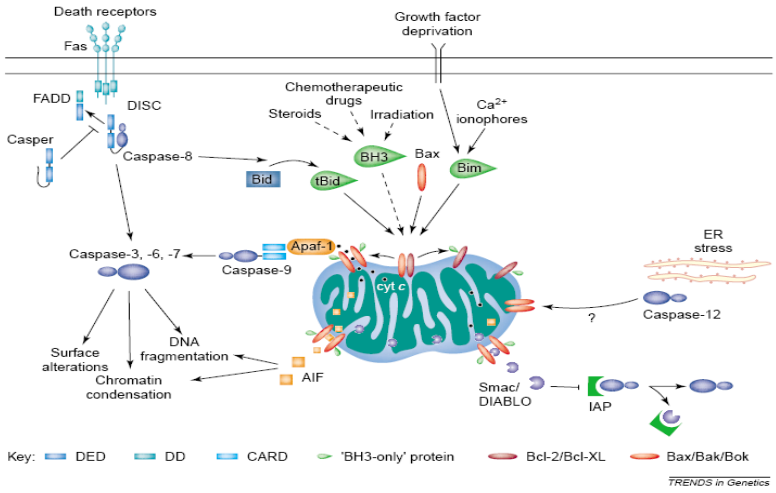


Figure II. 3: Overview of apoptotic signaling in mammalian cell; The explanation of basic apoptotic pathways is given in text; ad (Joza et al., 2002)

Smac/DIABLO is apoptosis regulator that is normally resident in mitochondria. After mitochondrial apoptotic pathway activation, it is released to the cytosol where it binds IAPs and thus hinders their inhibitory function (Joza et al., 2002). Blocking the release of Smac/DIABLO has been associated with TRAIL resistance in some, but not all, melanoma cell lines (Joza et al., 2002). TRAIL-resistant type II cancer cell lines compared with TRAIL-sensitive type II cell lines show reduced release of Smac/DIABLO and overexpression of Smac/DIABLO by transfection sensitized the resistant TRAIL-resistant type II cells to TRAIL (Zhang and Fang, 2005). Therefore Smac/DIABLO seems to be one of the major determinants of TRAIL sensitivity in these cells.

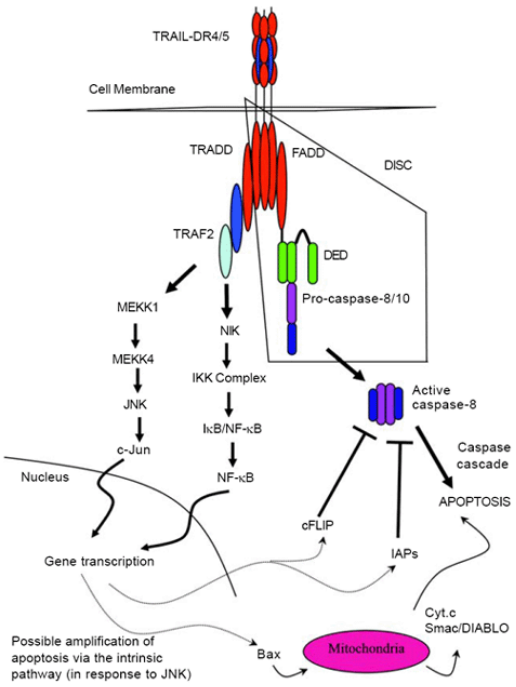


Figure II. 4: TRAIL receptor signaling. Ligation of TRAIL receptors (DR4 or DR5) usually triggers apoptotic pathway (trapezoid). Alternatively it can also lead to activation of NF-κB or c-Jun, which is both connected with anti-apoptotic effect via upregulation of pro-survival genes. ad (Aggarwal, 2003)

II.1.4 Physiological function of TRAIL

Physiological in vivo function of TRAIL-induced signaling was intensively studied in murine models. However, instead of two signaling human TRAIL receptors DR4 and DR5, mice have only one named mDR5 (murine death receptor 5), which is similarly

homologous to the human DR4 and DR5 (Wu et al., 1999). Thus the biological function of TRAIL signaling in mice does not have to fully reconcile its function in humans.

TRAIL$^{-/-}$ mice were viable, fertile and without any developmental defects and their examinations did not point to any crucial role for TRAIL in embryonic development (Sedger et al., 2002). However, their enlarged thymus raised former suspicion that TRAIL and its receptors have a role in the immune system (Sedger et al., 2002). Possibility that TRAIL has a role in immune surveillance of cancer has been extensively studied after Sedger et al. (2002) showed that a syngenic transplant of a B cell lymphoma grows much slower in TRAIL$^{+/+}$ mice and also forms less liver metastases than TRAIL-deficient mice. Later this anti-metastatic effect was assigned to NK cells and NKT cells that reduced the number of liver metastases in a TRAIL-dependent manner. TRAIL$^{-/-}$ and TRAIL-R$^{-/-}$ mice displayed higher frequency in developing spontaneous lymphomas at higher age (more than 500 days). Also tumors induced with the carcinogen methyocholantrene developed more often in TRAIL$^{-/-}$ mice (Cretney et al., 2002). However, loss of TRAIL-R did not accelerate incidence of lymphomas in p53-deficient mice (Yue et al., 2005). Hence, the TRAIL/TRAIL-R system at least in mice does not overtake of a tumor suppressor but apparently serves as one of the "weapons" of the immune surveillance that could be important for anti-metastatic defense. Importantly, as mentioned in the beginning of this chapter, TRAIL system could play much more important role in anti-tumor protection in long-lived mammals as humans.

In spite of mTRAIL mRNA expression in majority of tissues, there is no detectable mTRAIL on the surface of freshly isolated T cells, natural killer (NK) cells, NKT cells, monocytes, dendritic cells (DC) or B cells (Kayagaki et al., 1999). Nevertheless many innate immune cells as NK cells express mTRAIL after stimulation with interferon (IFN)-γ, IFN-α, IFN-β, interleukin (Il)-2, Il-15 or lipopolysaccharide (LPS) (Johnsen et al., 1999). Subset of hepatic NK cells constitutively expresses TRAIL in an autocrine IFN-γ-dependent manner (Zamai et al., 1998). Immature NK cells also express TRAIL and lose its expression after their maturation into granulated NK cells , whereas a small NK cells group does not undergo this maturation (Zamai et al., 1998). The persistent TRAIL-positive NK cells subset could then possibly serve for elimination of immature DCs, which are sensitive for TRAIL-induced apoptosis (Hayakawa et al., 2004).

TRAIL cell surface expression is increased on stimulated T cells. Anti-CD3 mediated activation of T_H1 but not T_H2 leads to upregulation of TRAIL and simultaneous increase of anti-TRAIL resistance in respective cells (Zhang et al., 2003). TRAIL signaling could also play a role in development of autoimmunity. Most of murine model-based studies describe a suppressive function of TRAIL on progress of artificially induced autoimmune diseases such as encephalomyelitis, diabetes or rheumatoid arthritis (Mi et al., 2003). Nonetheless, other autoimmune diseases as neuroinflammation may be accelerated by TRAIL (Aktas et al., 2005).

II.2 Cellular hierarchy

II.2.1 Stem cells

Stem cells are defined by their functional ability to (i) generate more stem cells and (ii) generate cells whose descendants differentiate into various cell types (Morrison and Kimble, 2006). The definition readily fits a newly fertilized egg, however even many adult organs contain stem cells (adult/tissue stem cells) that posses this unique capability called self-renewal (Qu and Shi, 2009).

Self-renewal is thought to exist in two forms. According to Yatabe (2001) about 95 % of the time a stem cell divides asymmetrically to leave one stem cell and one daughter cell that differentiates (=asymmetric self renewal). Sometimes (about 2,5 % of divisions) a stem cell produces one stem cell and one committed cell (=symmetric self-renewal); and the remaining 2,5 % of cases the stem cell becomes extinct as it produces two committed cells. Interestingly, it was shown that some cells seem to switch between both types of cell division according to needs of organism. This phenomenon was described e.g., in embryonic development or after injuries when stem cell pool expansion was required (reviewed in (Morrison and Kimble, 2006)). Hence, tissue stem cells apparently sit at the apex of tissue hierarchy, and are essential for maintenance of tissue homeostasis. Unfortunately, in contrast to embryonic stem cells the origin of adult stem cells is poorly understood. We also lack detailed information on the molecular programs and signaling governing these processes. Nonetheless, it is widely recognized that interaction of cells

with so called stem-cell “niche” is strongly involved in maintenance of stemness, and thus regulation of cell division and differentiation (Tulina and Matunis, 2001).

II.2.2 Tumorigenesis as a stem cell associated process

In the classical model of tumorigenesis by Nowell (1976), malignancy is viewed as a clonally-derived cell population, which acquires new potentially advantageous mutations that give rise to new more rapidly proliferating clones. Cell type which is most suitable for unlimited proliferation in the given environment then out-competes the less adapted clones in a process of clonal selection and becomes temporarily predominant (Nowell, 1976). This idea was based on genetic analysis of tumors at different stages of progression combined with the examination of subsets of clones within the tumors (Merlo et al., 2006). The source of apparent heterogeneity of cells within most tumors has been implicitly assigned to high instability of their genomes and micro-environmental influence.

However, novel insights in cancer and stem cell research, namely the recent discovery of stem cells in various adult organs and demonstration of their striking similarity to tumor cells, led to a renaissance of an alternative model that has been existing for decades. In this so called cancer stem cell (CSC) hypothesis, heterogeneity of tumor cell populations is explained by the possibility that tumor, analogically to a healthy tissue, is hierarchically organized (Hope et al., 2004). Cancer stem cells are then hypothesized to be a small subpopulation of cells sitting atop tumor hierarchy that are able to self-renew and undergo differentiation to generate various cell phenotypes observed in tumors. Importantly, according to CSC hypothesis, CSCs are the only cells of tumor that posses a capacity of unlimited growth, and are responsible for its progression, drug resistance, and recurrence; thus they present an ideal therapeutic target for a successful eradication of tumors (Ichim and Wells, 2006). Descendants of the CSCs, that have lost self-renewal capacity, can proliferate for a finite number of cell divisions before they ultimately differentiate to give rise to a much larger population of cells that can no longer proliferate.

Early observations suggesting that majority of cancer cells cannot proliferate continuously come from Mendelsohn (1960), who noted that only a portion of mammary

adenocarcinoma cells referred to as "growth fraction" incorporated ^{3}H-thymidine. However, pivotal experimental support for CSC theory came at first from acute myeloid leukemia in which a rare subset comprising 0,01-1% of total population could induce leukemia when transplanted to immunocompromised mice (Bonnet and Dick, 1997). Subsequently, evidence supporting the existence of CSCs was extended to solid tumors including melanoma, liver, lung, breast, prostate, brain, head and neck, pancreatic, mesenchymal and colon cancer (reviewed in (Visvader and Lindeman, 2008)). Unfortunately, limitations of xenograft assays used in these experiments confer to the fact that the proof of CSCs, at least for solid tumors, is highly controversial so far.

II.2.2.1 Origin of CSCs

The origin of CSC is still a subject of ongoing discussion[2]. Several interpretations of CSC hypothesis have emerged regarding this issue and some of them are not necessarily mutually exclusive. First, a tissue specific stem cell could be expected to be the target of transformation since the mitotically-active lifespan of differentiating cells seems too short for acquiring the necessary number of mutations (Jackson and Loeb, 1998). This opinion is supported by the observations that common signaling pathways regulate self-renewal in both normal and putative cancer stem cells (Al-Hajj and Clarke, 2004). Data obtained from analysis of promoter methylation showed that epigenetic profile of colorectal cancer samples, is similar to that of embryonic stem cells (Widschwendter et al., 2007). Further, Blair (1998) and others have shown that CSCs may share with their normal counterparts some immunophenotypic antigens.

[2]Since the CSC might be misleading denomination for these cells of uncertain origin and relation to normal stem cells, some authors proposed/preferentially use the term "tumor-initiating cell" (TIC) for cell capable of tumor re-initiation after its transplantation in immunocompromised mice (Neuzil et al., 2007). For purpose of this thesis, I use CSC nomenclature, so far more prevailing in literature. Moreover, the term TIC may evoke a perception that it refers to the cell from which the (original/primary) tumor derived in the first place. This is likely not the case since there is a clear evidence that the CSC or tumor initiating cell population can undergo changes as the disease progresses (Clarke, 2004).

Alternatively, CSCs may be neo-stem cells; that is, they may originate in a differentiating cell that has re-acquired through carcinogenesis the capacity for self-renewal. The latter possibility requires that acquisition of self-renewal occurs as a very early event in carcinogenesis, otherwise nascent cancer clones would be lost due to clonal extinction. Evidence for such reversal comes from experiments with MOZ-TIF2, MLL-ENL fusion proteins, which are able to restore self-renewal in myeloid progenitors (Huntly et al., 2004).

II.2.2.2 Markers of CSCs

Number of ways has been proved effective for the isolation of subsets enriched for CSCs. Especially various membrane proteins have been suggested as markers of CSC populations depending upon tumor type and tissue of which the tumor originates. As hinted above, markers specific for normal stem cells of a given organ may be useful for CSC identification in respective tumors. Clarke et al., (2003) was first to indentify a cellular population displaying CSC-properties in a solid malignancy. Only a subset of breast tumor cells positive for CD44 and negative for CD24 (CD44+ CD24-/low) were able to form a new tumor when injected into mammary fat-pad of immunodeficient mice. The expression profile of CD44+ CD24- CSCs was likewise shown to be more similar to the normal breast epithelial stem cells (Al-Hajj et al., 2003). Similarly other surface antigens e.g. efflux transporters ABCB5, ABCG2 and those of yet obscure functions such as CD34, CD90/Thy1, CD133 have been used as CSC markers i.a. in melanoma, tumors of breast, lungs, liver and brain respectively (reviewed in (Visvader and Lindeman, 2008)).

Expression of these and many other putative CSC markers is not absolutely tumor-exclusive and it is rather their combinations that are specific for particular tumor type. Moreover, CSC phenotype may not necessarily be uniform between cancer subtypes and one marker may be typical for several different tumors.

II.2.2.2.1 CD133/Prominin-1

For instance, apart from glioblastoma, CD133 has been reported as a CSC marker in medulloblastoma, pancreas, lung and colon cancers. In some other tumors, combination of

CD133 with one or more markers is used. In prostate cancer, subset of CD44+/α2β1high/CD133+ cells were shown to possess a capacity to self-renew and derive aberrantly differentiated cells that form the bulk of the tumor.

CD133 or human Prominin-1 is a pentaspan transmembrane glycoprotein of 865 aa and molecular weight 120 kDa, encoded by single copy gene on chromosome 4 (4p15.33). The CD133 molecule consists of N-terminal extracellular domain followed by 5 transmembrane domains and cytoplasmic C-terminal domain. The transmembrane domains are joined by two short intracellular loops of 30 aa each and two big extracellular loops of 255 aa and 290 aa respectively. Originally, CD133 was discovered as the target of monoclonal antibody (mAb), AC133, which was generated to bind CD34+ population of hematopoietic stem cells (Yin et al., 1997). Subsequently, it has been recognized as a suitable marker for stem cells of various origin e.g. fetal neural stem cells or renal, corneal and prostate tissue specific stem cells. It was later suggested, that not CD133 itself but rather its certain glycosylated epitopes, such as the one recognized by AC133, should be considered as "stemness" markers[3]. This was based on several discrepancies in identification of CSCs (Shmelkov et al., 2008). Several studies have demonstrated that expression of CD133 mRNA or protein is not restricted to stem cells and the AC133 epitope can be downregulated independently from CD133 during differentiation (reviewed in (Bidlingmaier et al., 2008)). Regrettably, very little is known about the molecular nature of recognized epitopes and their glycosylation. In fact, the exact location of the modified amino acid residues on CD133 targeted by AC133 and other commercial mAbs has not been described. An additional complexity ensues from alternative splicing that yields two known isoforms named AC133/1 and AC133/2 (Bidlingmaier et al., 2008). The shorter AC133/2 isoform, lacking 9 aa in the N-terminal portion, was shown be downregulated due to differentiation in basal epidermal stem cells. Thus, Yu (2002) concluded that AC133/2 but not AC133/1 is a marker of stem cells.

The biological function of CD133, its eventual ligands or intracellular interacting partners remain unclear. Whether and how CD133 *per se*, or its splicing and glycosylation forms contributes to the maintenance of primitive phenotype is questionable. However, its

[3] In this review I follow the notation of CD133+/- cells as it is used in original literature, even in the cases where experiments were done with commercial antibodies.

prominent localization in apical membrane protrusions and cholesterol-rich rafts is indicative of possible function in cellular polarization and signaling. Biological significance of CD133 was stressed by loss-of-function experiments, since mutation or absence of prominin-1 led to serious retinal defects and complete loss of vision (Maw et al., 2000).

II.2.2.2.2 ATP-binding cassette transporters

ATP-binding cassette (ABC) transporters constitute a family of five membrane proteins serving as efficient protection against DNA-damaging agents. These transporters are important for stem cells, due to role of stem cells in tissue regeneration, but are downregulated following differentiation. For example, ABCG2 (or breast cancer resistance protein 1 (BCRP1)), plays major role in drug resistance, exhibiting broad spectrum substrate specificity (Kusuhara and Sugiyama, 2007). Similarly, in melanoma ABCB5+ cells posses greater tumorigenic capacity than ABCB5- cells and have significantly higher capacity for self-renewal and differentiation (Schatton et al., 2008).

Identification of stem cells/CSC based on ABC transporters also involves subjecting of cells to DNA binding fluorescent dyes (e.g. Hoechst 33342) followed by FACS. This results in a distinct so called “side population” of cells marked by a low uptake of the dye (Zhou et al., 2001).

II.2.2.2.3 Other putative CSC markers

Besides membrane proteins, aldehyde dehydrogenase (ALDH) activity has been frequently used as a marker of both normal and cancer stem cells in many tumors and even cancer cell lines (Ginestier et al., 2007). Surprisingly, the ALDH1+ population in breast cancer shows only small overlap with the previously described CD44+CD24-/low phenotype (0,1-1,2%) (Al-Hajj et al., 2003). In this case, variation in marker expression could be attributable to culturing of cells before cell sorting rather than the use of fresh material; moreover phenotypic conjunction of CD44+CD24-/low and ALDH1+ yielded a

highly CSC-enriched population. Though, more careful examination will be surely needed to overcome present ambiguity of CSC markers, eventually, in order to design CSC-specific cancer therapy.

II.2.2.2.3.1 Sphere assay for identification of putative CSC

Sphere assay is a method originally developed for identification of normal stem cells. It was shown that normal adult stem cells form spheroid bodies when cultivated in non-adherent serum-free conditions and subsequently this setup was used for identification or enrichment of cancer stem cells (Galli et al., 2004). It remains however elusive, how and why are the spheres created. In addition, fact that both stem cells and progenitors may form spheres infirmed some previous inferences regarding stem cell-enrichment (Marshall et al., 2007).

II.2.2.3 Apoptosis and drug resistance of CSCs

Resistance of putative CSCs is usually correlated to the fundamental properties they share with normal SCs. Besides ABC transporter-mediated drug efflux and radioresistance due to activation of DNA-damage response (Bao et al., 2006), some other mechanisms have been recently discovered. For example caspase promoter hypermethylation was previously been linked to TRAIL-resistance (Hopkins-Donaldson et al., 2003)and a very recent study by assigned the TRAIL-resistance of SC-like glioma cells to the same mechanism (Capper et al., 2009). In a study by Zobalova (2008) CD133 positive MCF7 cells were resistant to TRAIL due to upregulated FLIP, and its silencing by siRNA lead to their sensitization.

II.2.2.4 CSC niche

Analogically to the situation with normal stem cells, stromal-epithelial interactions have been shown to be crucial in survival and differentiation of cancer cells (Cunha and Hom, 1996). Fibroblasts are a major constituent of the stromal compartment. The extent to which fibroblasts can directly influence the phenotype of individual cancer cells is not yet known. However, an integration of several recent observations suggests enormous hidden potential of this issue. Not only various cancer-associated fibroblasts (CAFs) were

suggested to promote tumor invasion (Qian et al., 2003), growth (Olumi et al., 1999) and production of angiogenic factors (Koshida et al., 2006); in a recent study done by Smetana et al., (2007) various fibroblasts derived from skin tumors were shown to induce dedifferentiation of keratinocytes.

CAFs were shown to secrete elevated levels of stromal cell-derived factor 1 (SDF-1) which trough its receptor CXCR4 activates signaling pathways related to motility, chemotaxis, and adhesion (Orimo and Weinberg, 2006). Interestingly, CXCR4 is also upregulated in stem cells, enabling their remarkable homing ability important for growth and development (Natarajan et al., 2006). Also another factor potentially regulating self-renewal of cells, BMP antagonist GREMLIN1 was observed in tumor niche of Scc (Sneddon et al., 2006).

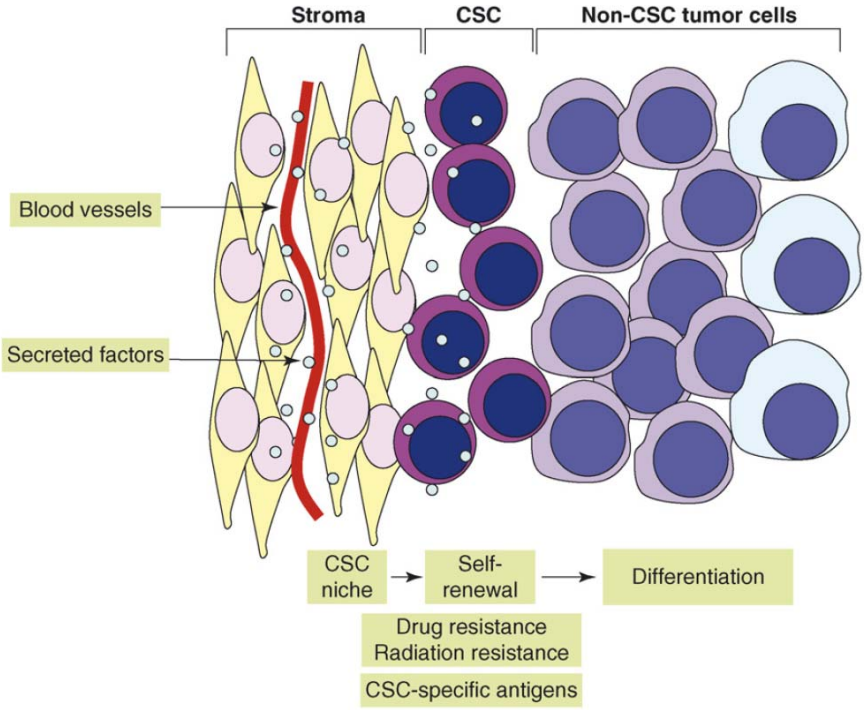


Figure II. 5: Role of tumor niche in solid tumor patogenesis. Tumor niche is created by blood vessels and fibroblasts. Niche renders support to CSC compartment, possibly enhancing its self-renewal and drug-resistance. CSCs self-renew and simultaneously generate large pool of (pseudo-)differentiated non-CSC tumor cells. ad(Ailles and Weissman, 2007)

III. Materials and Methods

III.1 Cell culture

III.1.1 Cell lines

All cells used in this study were derived from human healthy or cancerous tissues. The cell lines and primary cells used during this study are listed in **tab. III. 1**.

name	type of cells, tissue of origin	disease	stage	source
HCT 116	epithelial, colon epithelium	colorectal carcinoma	n/a	ATCC, USA
HT-29A3	secretory epithelial, colon epithelium	colon adenocarcinoma	Dukes' type C	Dr. E. Šloncová, IMG, ASCR
CX-1	secretory epithelial, experimental metastasis (nude mouse model)	colon adenocarcinoma	n/a	DSMZ, Germany
RKO	epithelial, colon epithelium	colon carcinoma	n/a	ATCC, USA
LoVo	epithelial, left supraclavicular regio	colorectal adenocarcinoma	Dukes' type C	ATCC, USA
Colo-206F	epithelial, ascites fluid	colon carcinoma	n/a	DSMZ, Germany
SW 620	secretory epithelial, lymph node	colorectal adenocarcinoma	Dukes' type C	ATCC, USA
SW 480	secretory epithelial, colon epithelium	colorectal adenocarcinoma	Dukes' type B	ATCC, USA
LS-174T	secretory epithelial, colon epithelium	colorectal adenocarcinoma	Dukes' type B	ATCC, USA
DLD-1	epithelial, colon epithelium	colorectal carcinoma	Dukes' type C	ATCC, USA
DLD-1E8	epithelial, colon epithelium	colorectal carcinoma	Dukes' type C	produced by lentiviral infection
BccF B3	fibroblasts, skin	basal cell carcinoma	n/a	Prof. K. Smetana Jr., Charles University in Prague, 1st Faculty of Medicine, Institute of Anatomy Prague, CR
Bfh B4	fibroblasts, skin	benign fibrous histiocytoma	n/a	
SccF S4	fibroblasts, skin	spinocellular carcinoma	n/a	
HF	fibroblasts, skin	no diagnosed disease	.	

Table III. 1: Cell lines and their specifications

For simplification following acronyms are used further in text: E8 for DLD-1E8; B3 for BccF B3; B4 for Bfh B4, S4 for SccF S4.

III.1.2 Culture media

III.1.2.1 Culture media for propagation of cells

HCT 116 and LS 174 T cell lines were cultured in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10 % FBS (*Hyclone*), non-essential amino acids (*Gibco*) and antibiotics (50 000 IU/l penicillin; 0.1 g/l streptomycin; 0.08g/l gentamycin; all *Sigma*).

Colo-206F, LoVo, SW 480 and SW 620 were cultured in RPMI supplemented with 10 % FBS (*Hyclone*) and antibiotics.

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS (*Hyclone*) and antibiotics was used to culture DLD-1, HT-29A3, CX-1, RKO cell lines and SccF S4, Bfh B4, BccF B3, and HF primary fibroblasts.

DLD-1E8 cells were maintained in selection media to ensure maintenance of expression plasmids, ***i.e.*** DMEM with 10 % FBS (*Hyclone*) and standard antibiotics supplemented with 3 μg/ml puromycin (*Sigma)*. All above mentioned media are further also referred to as "regular" or "recommended" media.

III.1.2.2 Serum free medium for cultivation of spheroids (NS-Ac)

For cultivation of colon carcinoma cell lines in serum-free conditions (or sphere assay - SA) NeuroCult® NS-A proliferation medium *(StemCell Technologies)* was used. This medium was mixed from its components prior to each cultivation experiment, since single components are declared unstable and therefore inconvenient for long term maintenance in unfrozen state. Serum-free medium containing supplementation was stored in a dark place, at 4 °C, and has never been in use for longer than 1 week. We designated this medium "NS-A complete" (NS-Ac) and it was prepared as follows:

NS-A basal medium and NS-A proliferation supplement[4] *(both StemCell Technologies)* were thawed overnight at 4° C and mixed in 9 : 1 ratio in amounts sufficient for the respective experiment. Stock solutions of Heparin, human epidermal growth factor

[4]Contents of the commercial proliferation supplement is not provided by manufacturer. We estimate its character from analogous mixture for amplification of tumoral stem cells patented in WIPO under notation WO 2006/030473 A1.

(EGF) and human basic fibroblast growth factor (bFGF) *(all Sigma-Aldrich)* were thawed just before the media utilization and added media to reach final concentrations of 2 μg/ml heparin, 20 ng/ml EGF, 10 ng/ml bFGF.

III.1.3 Propagation, freezing and thawing of cells

All primary cells and cell lines were grown in Petri dishes, cell culture flasks or multiwell plates with standard tissue culture treated surface *(TPP)*. Cultivation was carried out in incubators (*Sanyo*) at 37 °C, 5 % CO_2 (standard cultivation conditions). All work was done using sterile equipment and standard tissue culture techniques. Cell cultures were regularly checked for the presence of mycoplasma.

The proliferation intensity of each cell line was acquired by counting its doubling time. Fresh cells (not sooner then 2 days but maximum 1 week after thawing) were harvested, counted in hemocytometer, diluted in fresh media to uniform low concentration of 30000 cells/ml and seeded in 6-well cell culture plates. Cells were incubated in standard conditions (as described previously) for 48 hours, then harvested and counted. The doubling time T is then calculated as follows: $T = t * \ln(2) / \ln(c_2/c_1)$ where t is time of incubation, c_2 is final and c_1 initial concentration of cells.

In case of primary fibroblasts, record of passages and population doublings was kept, and cells were not used when suspected of nearing senescence.

Freezing of cells was performed in regular culture media with 20 % FBS and 10 % dimethylsulphoxide (DMSO) *(Sigma-Aldrich)*. Cells in cryotubes *(Nunc)* were stored in freezing containers *(Nalgene)* at – 80 °C overnight and subsequently moved to liquid nitrogen (- 196 °C).

Thawing cells was done rapidly in liquid bath (37 °C). Cells were diluted into medium, spun down, and then resuspended in warm regular medium. Viability of cells was assesed by trypan blue staining and was mostly between 65-95 %.

III.1.3.1 Cultivation of cells in serum-free conditions

Before seeding in serum-free medium, cells were harvested, counted, centrifuged at 250g, 4 °C, 5 minutes, *(centrifuge 5810 Eppendorf, Germany)*, washed with PBS to remove residual serum and again centrifuged. Cells were resuspended in NS-Ac and seeded in clonal densities to either tissue culture treated *(TTP)* or non-treated *(Nunc)* multiwell plates.

III.1.3.1.1 Subculturing spheroids

For subculturing spheroids we used protocol for subculturing human neurospheres available at http://www.stemcell.com/technical/28724_Neurocult%20Human.pdf . For poor performance with plain trituration we extended the procedure for trypsinization step. Briefly: cells were spun in u-bottom multiwell plates or microtubes and supernatant was discarded. Cells were treated with trypsin-EDTA solution *(Sigma-Aldrich)*, 10 minutes, at 37 °C and repeatedly triturated trough pipette tips (circa 100x) until the single cell suspension was reached. Trypsinization was terminated by adding stechiometraical amount of soybean trypsin inhibitor *(Sigma-Aldrich)* to the suspension. Suspension was spun and washed twice with PBS. Cells were counted and resuspended in fresh NS-Ac medium. Importantly, during comparative experiments all cells were subcultured accordning to this protocol, including the non-spheroid cell lines and cells in regular media were treated analogically. Identical steps were also used for harvesting of cells prior to processing for analysis on flow cytometry.

For minimizing exposure of cells to such burdening manipulation, experiments were designed to evade repeated subculturing. Cells were plated in low densities regarding their proliferation rates to obtain approximately equal cell counts in all tested cell lines at the time of processing.

III.2 Treatment of cells with TRAIL

For induction of apoptosis, recombinant TRAIL Killer (Apronex) was diluted to appropriate concentration in prewarmed respective media and added to incubation with cells for required time. Stock of TRAIL was divided into small aliquots (before storing at -80 °C) to prevent repeated freeze-thaw cycles, thus maintain constant quality of used reagent.

III.3 Flow cytometry

III.3.1 Measuring of apoptosis

We used two flow cytometric approaches for measuring of apoptosis utilizing intracellular and superficial staining of apoptotic cells.

III.3.1.1 Annexin V FITC staining

This procedure is based on loss of membrane phospholipid assymetry and exposure of phosphatidylserine (PS) in apoptotic cells. Cells are not fixed for this type of staining.

Cells were harvested according to procedures described above and maintained in microtubes on ice. Cells were washed twice with ice cold PBS then resuspended in ice cold annexin V binding buffer (ABB) 1x *(Apronex)* and incubated 10 minutes on ice. Cells were spun, transferred in ice cold ABB 1x with annexin V FITC *(Apronex)* diluted to final concentration 0,5 μg/ml and incubated in dark place on ice for 30min. Cells were spun, washed with ice cold ABB 1x and resuspended in ABB 1x with propidium iodide (PI) *(Sigma-Aldrich)* diluted to final concentration 20 μg/ml. Samples were stored in dark until analysis by flow cytometry.

Where not explicitly stressed, all washing (and transfering) steps were followed (and preceded) with centrifugation at 250 g, 4 °C, 5 minutes.

III.3.1.2 M30 FITC staining

This procedure is based on detection of caspase-cleaved cytokeratin-18 in apoptotic cells. Cells are fixed for this type of staining.

Cells were harvested according to procedures described above and maintained in microtubes on ice. Cells were washed twice with ice cold PBS then resuspended in ice cold methanol and fixed for 30 minutes in freezer at -20 °C. Cells were washed twice with PBS and resuspended in incubation buffer (PBS + 1% BSA) with M30 FITC conjugate *(Roche)* diluted to final concentration 0,5 μg/ml. After 30-min incubation in dark place at room temperature cells were washed twice with PBS and resusspended in PBS. Samples were stored in dark until analysis by flow cytometry.

Where not stressed, all washing (and transfering) steps were followed (and preceded) with centrifugation at 250 g, 4 °C, 5 minutes.

III.3.2 Profiling of surface proteins

Cells were stained with directly conjugated antibodies or sequentially, with primary then secondary antibody in appropriate combiantions. Monoclonal antibodies used for analyses of death receptors and CSC markers are listed. Dead cells were excluded from analysis by addition of Hoechst 33258 *(Sigma-Aldrich)*.

target	mAB	host / isotype	*Manufacturer*
DR4	MAb to TRAIL-R1 clone HS1010	mouse / IgG1	*ALEXIS Biochemicals*
DR5	MAb to TRAIL-R2 clone HS201	mouse / IgG1	*ALEXIS Biochemicals*
Fas (CD95)	clone DX2	mouse / IgG1	*R&D Systems*
TNFR I	clone 4.12	mouse / IgG2a	*Invitrogen*
CD133/1	clone AC133 PE conjugate	mouse / IgG1	*Miltenyi Biotec*
CD133/2	clone 293C3 PE conjugate	mouse / IgG2b	*Miltenyi Biotec*
ABCG2	clone 5D3	mouse / IgG2b	*R&D Systems*
mouse IgG	115-115-164 PE conjugate	goat / whole IgG	*Jackson Immunoresearch*

Table III. 2: List of used antibodies

Surface antigen staining protocol (sequential staining):

Cells were harvested, centrifuged, washed with cold PBS resuspended in incubation buffer (PBS + 0,2 % gelatine *(Sigma-Aldrich)* and 0,1 % sodium azide *(Sigma-Aldrich)*) and maintained on ice. For blocking of eventual Fc-receptors normal human AB serum

(ExBio) was added to incubation for 10 minutes, then the cells were spun, washed with cold PBS and resuspended in incubation buffer with diluted primary antibody (1 μg/ml). Cells were incubated with primary antibody 30 minutes on ice, then spun, washed twice with cold PBS and resuspended in incubation buffer with PE-labeled secondary antibody (1 μg/ml), stored in dark place on ice and incubated for 45 minutes. Cells were spun, washed twice with cold PBS and resuspended in PBS with Hoechst33258 vital dye *(Sigma-Aldrich)* (5 μg/ml). Samples were stored in dark until analysis by flow cytometry.

Where not stressed, all washing (and transfering) steps were followed (and preceded) with centrifugation at 250 g, 4 °C, 5 minutes.

Direct staining (with AC133-PE and 293C3-PE conjugates) was performed analogically with omitting the first staining step.

III.3.3 Cytometer setup

For all flow cytometric analyses, LSRII instrument *(BD Bioscience, USA)* was used. Samples were prepared in u-bottomed 96-well plates and loaded with HTS peripheral device of the cytometer.

Gating of vital i.e. non-necrotic population was enabled by co-staining with vital dyes (PI in annexin- FITC apoptosis assays, Hoechst 33258 *(Sigma-Aldrich)* in PE staining). Examples of gating are given in respective figures in results chapter.

III.4 Caspase 3/7 glo assay

Caspase 3/7 glo luminiscence assay *(Promega)* was performed as alternative method for assessing apoptosis. Since this assay measures the activity of enzymes in cellular lyzates and is purely quantitative, cells were carefully counted for every caspase 3/7 assay experiment. Cells must be plated on non-transparent white background for this assay; therefore duplicate with ordinary transparent plate was always simultaneously created to visually control the cell counts.

Procedure was done according to manufacturer's protocol, which is available at: http://www.promega.com/tbs/tb323/tb323.pdf

Luminescence was measured in duplicates on Luminoskan Ascent instrument *(Thermo Scientific, USA)* and background values of media were subtracted.

III.5 Co-cultures with stromal fibroblasts

Two approaches were used in co-cultures of colon carcinoma cells with fibroblasts (co-cultures with conditioned media, and direct co-cultures). In case of direct co-cultures, subsequent flow-cytometry required to distinguish the cells with fluorescent labels.

III.5.1 Preparation and application of fibroblast-conditioned media

We used all four fibroblast types (**tab. III. 1**) for conditioning media for cultivation of colon cancer cell lines. Briefly: fibroblasts were grown to confluence. Their partially exhausted medium was replaced with fresh regular DMEM. DMEM was harvested after one day and added to colon cancer cells in 1:1 ratio with their respective fresh medium.

III.5.2 Direct co-cultivation of colon cancer cells with fibroblasts

III.5.2.1 Labeling of cells with CellTracker Red (CMTPX)

CMTPX dye *(Invitrogen)* was used for staining of fibroblasts according to instructions of manufacturer. Briefly: the working solution of CMTPX was prepared by dissolving lyophillizated product in DMSO to concentration 10 mM and subsequently adjusting to final concentration 1 μM by dissolving initial stock in serum-free DMEM. Cells were grown in a multiwell plate to confluence. Medium was removed from the plate and prewarmed CMTPX working solution was added to the cells. Cells were incubated for 30 minutes under standard conditions, then working dye solution was replaced with regular DMEM with 10 % FBS and incubated for another 30 minutes. During this time, according to manufacturer's claims, the dye underwent chemical change (reduction) that prevents it from escaping intracellular compartment. Redundant dye or dye that failed to undergo reduction stayed in the supernatant. Medium was replaced again with the fresh prewarmed

regular DMEM with 10 % FBS. Since then, cells were cultured normally and were prepared for co-culture.

III.5.2.2 Labeling of cells with RFP

For labeling of cells with RFP we employed lentiviral infection approach. We generated stable trasfectants of colon carcinoma cell lines expressing RFP.

III.5.2.2.1 Lentivirus production

This work was kindly done by Dr. Anděra and Dr. Koc according to following protocol: http://mellgenlabs.com/Documents/ezlentifect_protocol.htm RFP expressing lentivirus vector was used *(Hebrew University of Israel)*.

III.5.2.2.2 Infection of target cells

Cells were grown in 24-well plate to 60% cell confluency. For one well 15 µl of concentrated virus supernatant was dropped into 0,5 ml of medium and incubated for 24h After that cells were washed 2 times the with PBS and let in the fresh medium with selective antibiotics (2 µg puromycin per ml of medium) for 24 h. Next day the medium was replaced and stronger doses of selective antibiotics added (3 µg puromycin per ml of medium).

III.5.2.2.3 Selection of RFP- expressing clones

RFP-expressing mixed cultures were diluted to very low clonal densities (approx.10 cells/ml) in regular media with puromycin (3 µg/ml) and seeded on 10cm Petri dishes. After 14 days of cultivation in standard conditions, individual colonies with the brightest expression of fluorescent protein were picked in sterile conditions under fluorescence macroscope Z16 APO A *(Leica, Germany)* and placed in wells of 96-well plate with regular media and puromycin (3 µg/ml). Cells were subsequently expanded by regular cultivation in the continuous selection conditions to obtain sufficient amounts of

cells for cryopreservation in liquid nitrogen. Before freezing, cells which retained expression of fluorescent protein were characterized for their sensitivity to TRAIL-induced apoptosis (**fig. IV. 10 a)**).

III.5.2.3 Seeding of cells for direct co-cultivation

For all co-cultivation experiments, fibroblasts were seeded first to create a confluent feeder layer on the bottom of the plastic wells. Colon cancer cells (DLD-1E8, DLD-1, HCT 116) were diluted in regular media and seeded in low densities into plates with fibroblast feeders (**fig. IV. 10 b)**) Dilution of cells was calculated for cells according to their proliferation rates to obtain near sub-confluence before processing for analysis. For recording cellular morphology and expression of fluorescent protein, we used solely inverted fluorescence microscope DM 6000B with integrated camera *(Leica, Germany)*.

IV. Results

IV.1 Initial characterization of used colon carcinoma cell lines

Colorectal carcinomas represent one of most often diagnosed and deadly cancers in the population of mainly Western countries and were also reported to comply (at least in some reports) with the cancer stem cells (CSC) model of tumorigenesis(Ricci-Vitiani et al., 2007). Therefore we in our experiments related to cancer stem cells rather intentionally focused on the analysis of colorectal cell lines, and in this study we used ten colon carcinoma cell lines of distinct origin and phenotypes. We started the experiments assessing basic properties of the cells including their doubling times, morphology and sensitivity to apoptosis induced by TRAIL. The analysis of the cell surface expression of death receptors and CSC markers was part of later experiments addressing phenotype of colon carcinoma cells depending on various cultivation conditions.

IV.1.1 Morphology of colon cancer cells

With a single exception of Colo-206F, all cell lines grew adherently in standard cultivation conditions and showed mostly uniform flat (HCT 116) to spindle-like (SW 480) morphology (**fig. IV. 1a), b)**). Colo-206F grew semi-adherently with a large number of floating or weakly adherent cells (**fig. IV. 1c)**), and this growth mode was not associated with a reduced viability.

When grown adherently, HT-29A3 cell line slightly tended to aggregate and form irregularly shaped cloudlets even low cell density, whereas other colon carcinoma cells formed multiple layers with dome detached cells only at higher densities (**fig. IV. 1d)**)..

a) **b)** **c)** **d)**

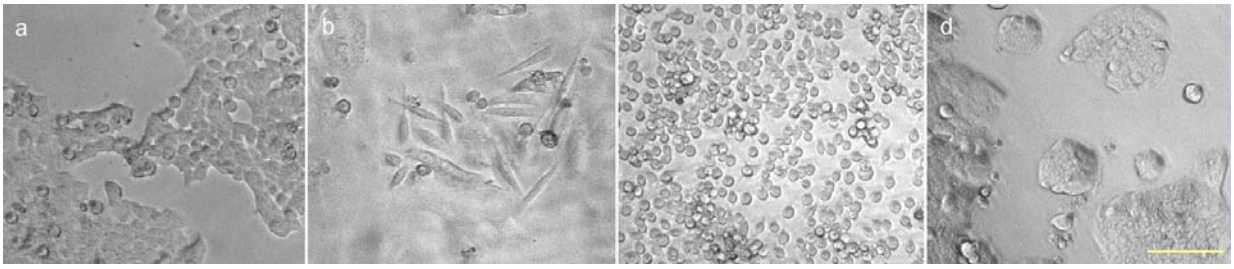
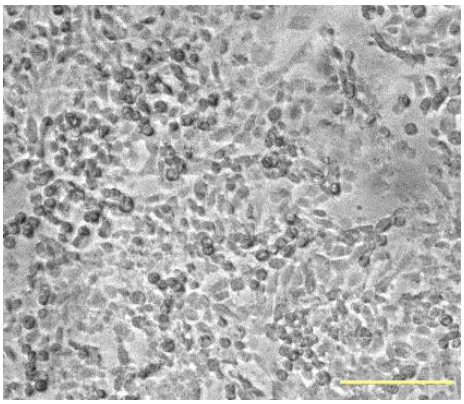


Figure IV. 1: Morphology of colon cancer cell lines. Cells were cultured in their recommended media for one week. Photographs were taken two days after the last passage, time sufficient to allow adherence. **a)** squamous cells (HCT 116) **b)** spindle shaped cells (SW-480) **c)** semi-adherent/floating cells (Colo-206F) **d)** compact cloudlets of cells (HT-29A3). Yellow bar represents 100μm and is common for **a), b), c), d)**.

IV.1.2 Colon cancer cells proliferation

We calculated the doubling times of given cell lines and the values were spanning from as high as 41,6 hours for Colo-206F cells to the lowest in HCT 116 and DLD-1 cells that doubled their populations in 17,7 and 17,1 hours respectively (**fig. IV. 2 b)**). Colon carcinoma cell lines showed generally lack of contact inhibition and were capable to continue growth even after reaching monolayer (**fig. IV. 2 a)**) .

a)



b)

Cell line	Doubling time in common medium [h]	σ
HT-29A3	35,0	2,1
LoVo	23,8	1,8
Colo-206F	41,6	2,6
SW 480	40,9	3,4
HCT 116	17,7	0,9
DLD-1	17,1	1,1
LS-174T	26,2	2,2
RKO	19,0	0,9

Figure IV. 2: Proliferation of colon cancer cells. a) HCT 116 colon carcinoma cells grown in culture for 5 days without passaging. After reaching confluence, cells continued to grow in more layers. Yellow bar represents 100μm. **b)** Doubling times of cell lines calculated from cell count increments after 48 hours in culture. All cell lines were sub-confluent at the time of final cell counts evaluation. The estimation of doubling time for each cell line was repeated several times and the average values with standard deviations are shown.

IV.1.3 Sensitivity of colon cancer cells to TRAIL-induced apoptosis

Next we examined whether and how are cells from this panel sensitive to TRAIL-induced apoptosis induced with the recombinant TRAIL under our standard cultivation conditions. For the detection and quantification of TRAIL-induced apoptosis we used AnnexinV-FITC-based flow cytometry analysis. Cells were treated with six different concentrations of TRAIL diluted in corresponding cultivation media. To reveal the kinetics of apoptosis, the experiment was parallelly performed at two time points, by adding the TRAIL in a reverse manner 15 and 6 hours prior to Annexin V-FITC staining and flow cytometric analysis.

While RKO cells were markedly resistant to even high concentrations of TRAIL, other cell lines demonstrated moderate to high sensitivity beginning at TRAIL concentrations as low as 20 ng/ml. The average values of FITC-positive fraction of technical duplicates are summarized in **fig. IV. 3 a)**

In addition to the Annexin V-FITC staining of the phosphatidyl serine externalized on apoptotic cells we used Caspase-Glo 3/7 Assay as additional methods for the quantification of TRAIL-induced apoptotic signaling. This method is based on the quantification the activated effector caspases and reflects the initial efficacy or robustness of their undirect activation by TRAIL. Activaty of the effector caspases measured after 3 hours of incubation with TRAIL and in principle followed the sensitivity profiles obtained by flow cytometry. Low sensitivity of RKO in comparison with other cell lines was confirmed. In addition caspase test revealed low activation of caspases after 3 hours of treatment in HT-29A3 cell line.

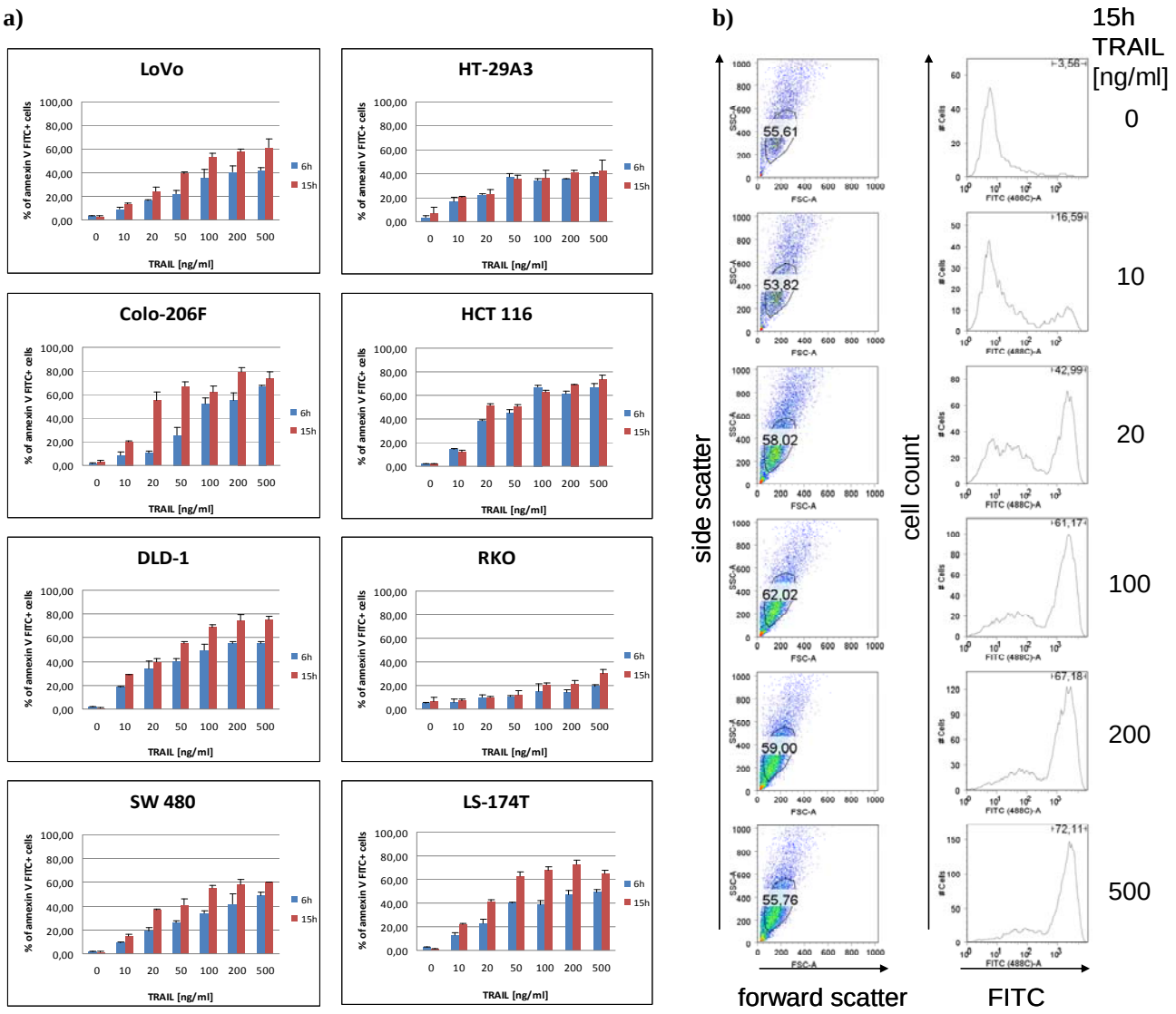


Figure IV. 3: Apoptosis of colon cancer cell lines cultivated in standard conditions (flow cytometry).

a) Eight colon carcinoma cell lines were grown under standard conditions in corresponding cultivation media. TRAIL diluted in the respective media to concentrations from 10 ng/ml to 500 ng/ml was added to sub-confluent cells 15 or 6 hours prior to processing of cells for flow cytometry. Blue and red bars represent averages of annexin V FITC+ fractions of technical duplicates of 6-hour treatment and 15-hour treatment respectively. **b)** Example of gating and processing raw FACS data. Left: debris and conglomerates are excluded in scatter dot-plot, single cells (gate) are taken for further evaluation, (LoVo cells, annexin V FITC staining).

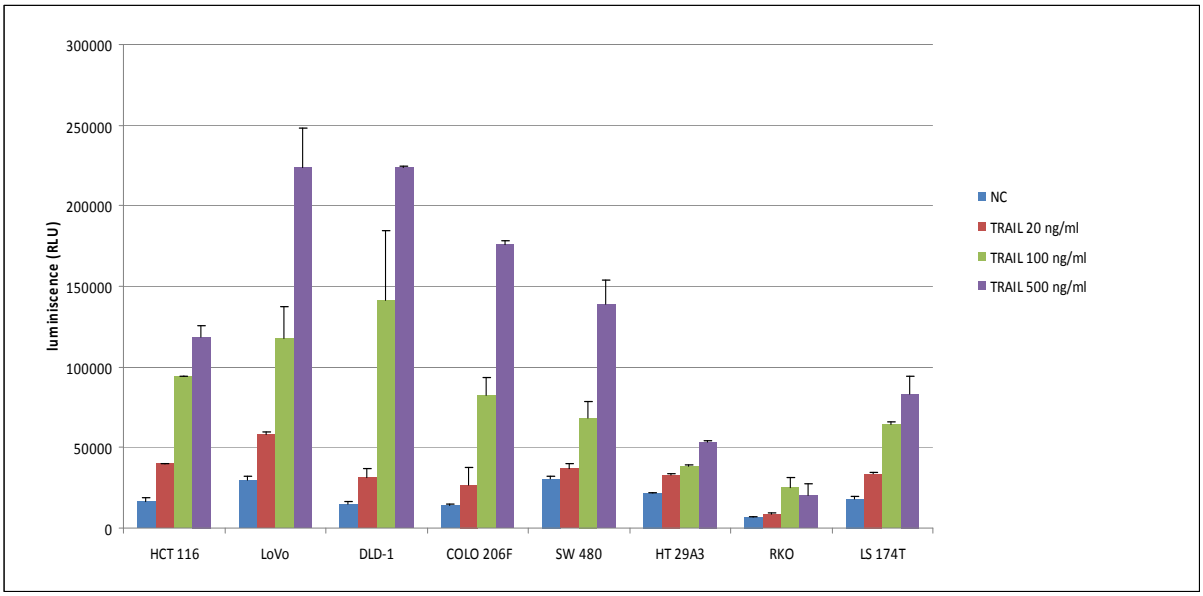


Figure IV. 4: Apoptosis of colon cancer cell lines cultivated in standard conditions (caspase 3/7 assay). Eight colon carcinoma cell lines were grown under standard conditions in their recommended media. TRAIL diluted in the respective media to concentrations from 20 ng/ml to 500 ng/ml was added to sub-confluent cells 3 hours prior to processing of cells for luminescence-based quantification of caspase 3/7 activation. Bars represent averages of relative luminiscence units (RLU) of biological duplicates.

IV.2 Influence of cultivation conditions on the behavior of colon carcinoma cell lines

IV.2.1 Morphology of cells is mostly changed in NS-Ac serum-free defined medium

Cultivation of breast and other cancer cells in the defined, serum-free medium, originally introduced to support growth of neural stem cells (NS-Ac), allows formation of so-called spheroids and was reported to allow preferential growth of less differentiated, progenitors or stem cells resembling tumour cell cultures (Ricci-Vitiani et al., 2007). We used this growth conditions for our panel of colorectal cell lines and compared it (both morphologically and functionally) with their cultivation under the standard conditions. We have observed significant shift in morphologies of all studied cell lines cultivated in NS-Ac medium. In general, cells tended to aggregate, while simultaneously in most cases, their adherence to surface was compromised. Consequently, increased number of floating of

cells and switching of shape from spindle to round was observed in several cell lines. When seeded at clonal concentrations, cells stayed together as they proliferated, and eventually adhered to other growing clones in their proximity.

Particularly two cell lines, HCT 116 and HT-29A3, (**fig. IV. 5 a)**) showed a prominent ability to form compact spheroid bodies. Unlike other clusters, spheroids were compact, rounded and almost uniformly sized formations which emerged all at once, usually on day 2 to 4 of cultivation in NS-Ac, depending on initial cell concentration.

In some prolonged serum-free cultivations, we observed rare spheroid-like clusters even in other cell lines including LoVo, COLO-206F and SW 480 (**fig. IV. 5 b)**). Since the definition of spheroid body is arbitrary, they might represent either the same entity or artifact.

IV.2.1.1 Contributions of selected components from NS-Ac media to morphologic changes of cultivated cells

To determine which component of the NS-Ac media is pivotal for these dramatic morphological changes of examined cells, we used different combinations of NS-Ac components, serum and cultivation plastic labware. Except for Colo-206F, usage of non-cell culture-treated dishes did not seriously compromised cell adherence. The only factor necessary but not sufficient for enabling spheroid growth was the commercial proliferation supplement most likely containing (apotransferrin, insulin, putrescin, selenium, and progesterone) While supplementation with EGF and bFGF was not necessary for spheroids initiation itself, it enhanced their growth, resulting in larger diameter of the final spheres (**fig. IV. 5 c)**).

When added, serum caused reversal to normal adherent phenotype in all cases, irrespectively of presence of proliferation supplement, growth factors and heparin (data not shown).

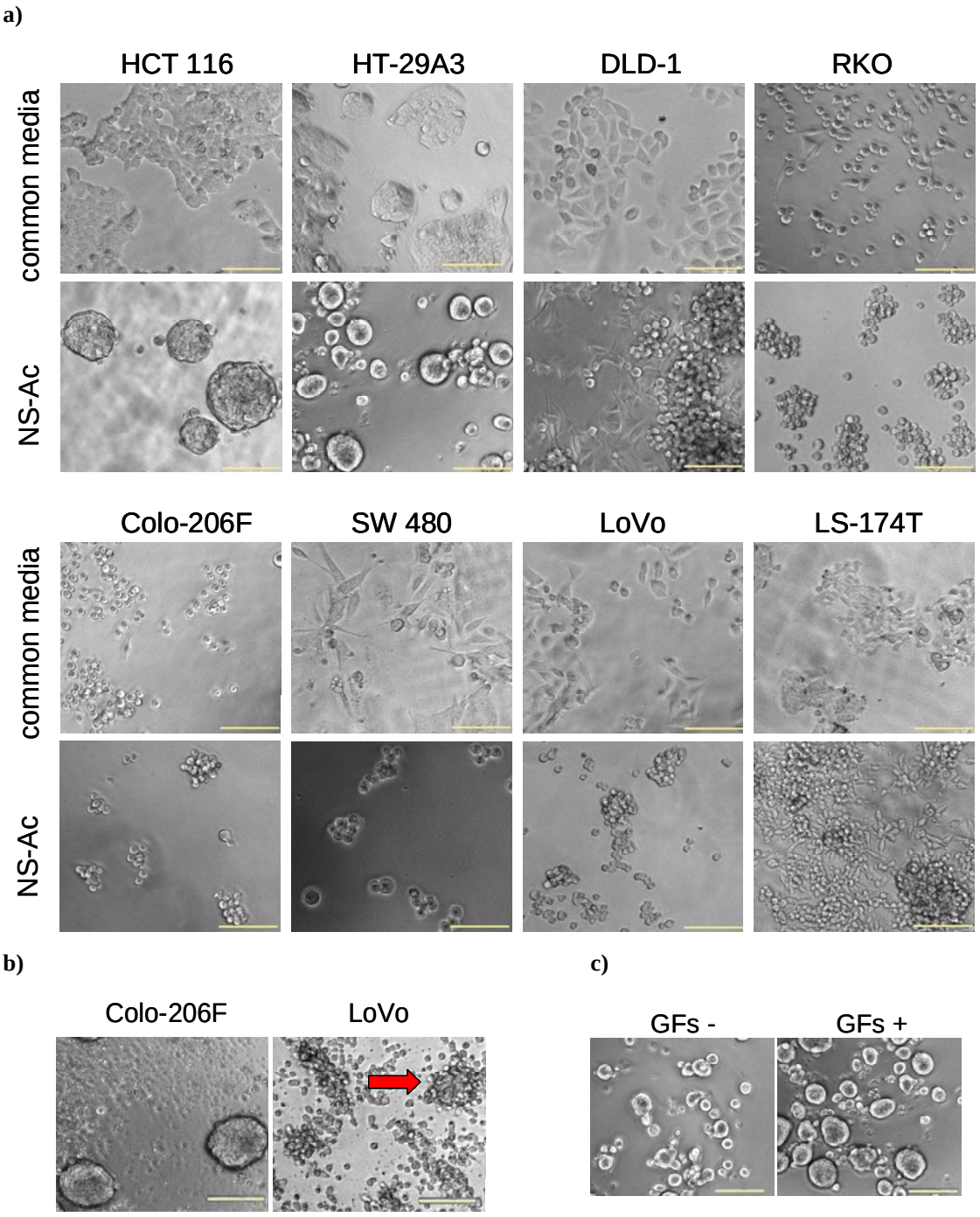


Figure IV. 5: Changes in cellular morphologies of colon cancer cell lines during cultivation in NS-Ac medium. a) Cells were cultured in the regular versus NS-Ac medium for six days. Passages were performed when needed to prevent their confluence. Upper rows in both portions show normal phenotype, lower rows show modified phenotypes in NS-Ac medium. **b)** Colo-206F and LoVo cell lines formed rare spheroids when cultured in NS-Ac for prolonged periods (two weeks). Left: Colo-206F shperoids are surrounded by rest of cells and cellular debris. Right: red arrow points to putative LoVo spheroid. **c)** Effect of EGF and bFGF supplementation on spheroids (HT-29A3, 6 days in NS-A). Yellow bar represents 100μm in **a), b), c)**.

IV.2.2 Cultivation in NS-Ac medium attenuates proliferation of colon carcinoma cell lines

Side-by-side comparison of cells cultivated under the standard and serum-free (NS-Ac medium) conditions indicated that their proliferation in the NS-Ac serum-free medium might be attenuated. Calculation of the doubling times revealed their averaged 25 % increase in the population doubling time, which was most apparent in fast proliferating and sphere forming HCT 116 cells (45% increase) (**Figure IV. 6**).

Cell line	Doubling time in regular medium [h]	Doubling time in NS-Ac medium [h]	Increase
HT-29A3	35,0	42,1	20 %
LoVo	23,8	28,7	21 %
Colo-206F	41,6	48,6	17 %
SW 480	40,9	54,0	32 %
HCT 116	17,7	25,7	45 %
DLD-1	17,1	21,7	27 %
LS-174T	26,2	33,0	26 %
RKO	19,0	23,5	24 %

Figure IV. 6: Doubling times of colon cancer cells cultivated in the regular and serum-free medium. Doubling times of cell lines calculated from cell count increments after 48 hours in culture. All cell lines were sub-confluent at the time of final cell counts evaluation. Experiments were repeated several times with similar results.

IV.2.3 Serum-free cultivation effect on TRAIL-induced apoptosis

We employed M30 FITC flow cytometry approach to measure TRAIL-induced apoptosis, resp. TRAIL-induced activity of the effector caspase-3 of colon carcinoma cell lines grown in the serum-free versus regular cultivation media. While in majority of cell lines their cultivation in NS-Ac medium compromised their sensitivity to TRAIL, in others (LoVo, Colo-206F) the effect was opposite. RKO were exceptionally negative for M30 FITC (presence of caspase-3-cleaved cytokeratin 18) signal, and were thus considered principally immeasurable by this method (**fig. IV. 7 a)**).

Next we decided to verify sensitivity of cells to TRAIL-induced apoptosis after differential cultivation by annexin V FITC staining. For this experiment, we chose three

highly to moderately TRAIL-sensitive cell lines, DLD-1, HCT-116 and HT-29A3, regarding their previously observed distinct capability of forming spheroid clusters. Annexin V-FITC staining of the analyzed cells confirmed the data obtained using the M30 FITC assay (**fig. IV. 7 b)**).

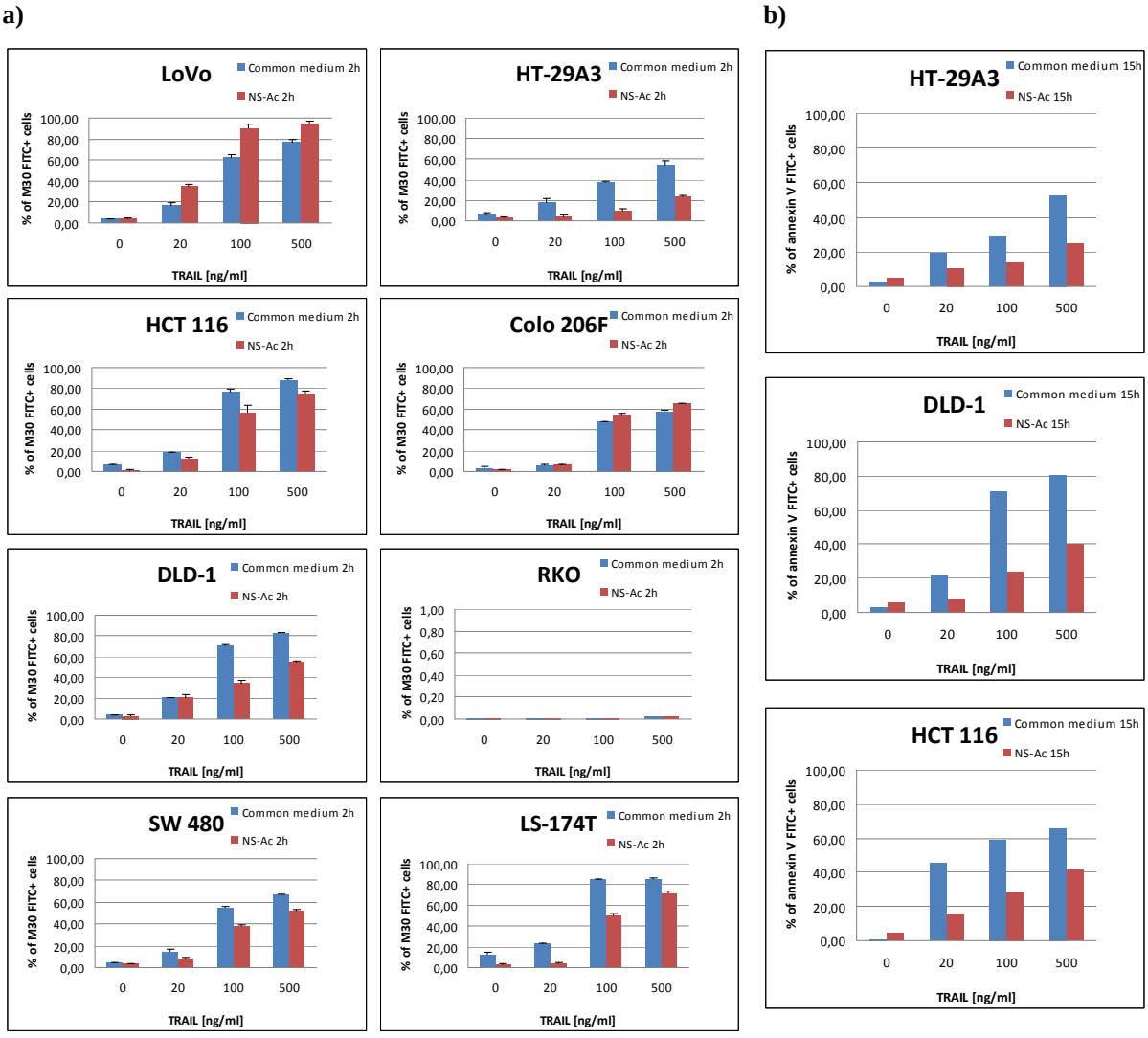


Figure IV. 7: TRAIL-induced apoptosis of colon cancer cell lines grown under different cultivation conditions. **a)** M30 FITC flow cytometric evaluation of analyzed cells was carried out after their 2-hour incubation with TRAIL. Cells were parallelly cultured in their regular media (blue bars) or NS-Ac (red bars) for six days. Bars represent averages of technical duplicates. RKO cells were repeatedly proved to be negative in M30 FITC assay. **b)** Annexin V FITC flow cytometric verification of differential TRAIL sensitivity of selected cancer cell lines. All cells were sub-confluent at the time of processing in both experiments.

IV.2.4 Cell surface receptors profiling of colon cancer cells grown under different cultivation conditions

Changes in growth media implemented TRAIL-induced apoptosis of colon cancer cells might be related to changed expression of its proapoptotic receptors. In addition these different cultivation conditions might affect cell surface of additional, stem cells-related markers. Thus in the next set of experiments we undertook comparative analysis of their expression.

IV.2.4.1 Expression of CSC markers

By means of PE labeled mABs staining and subsequent flow cytometry, we measured surface expression of CD133 and ABCG2, proteins commonly associated with CSC immunophenotype.

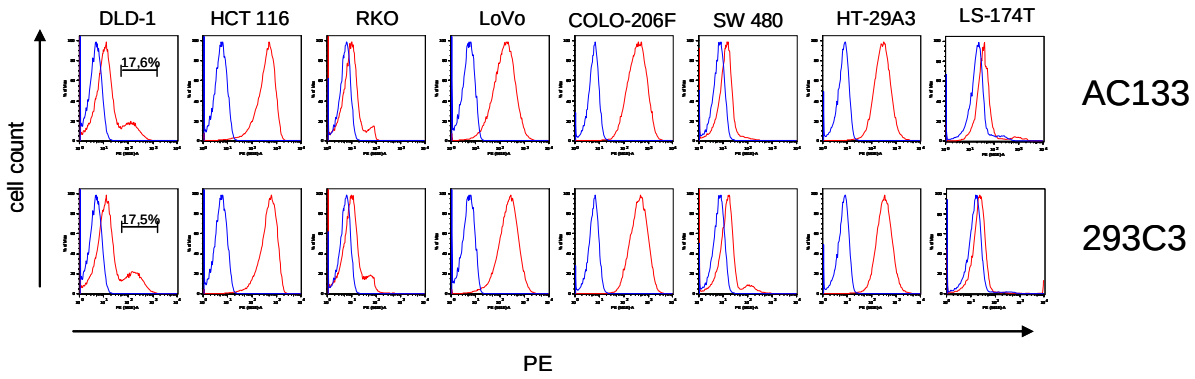
We initially performed comparison of two commercially available conjugates AC133 and 293C3, recognizing two reputedly distinct glycosylated CD133 epitopes, to determine their utilization in following analyses. Minimum difference was found between staining of cells surface expressed CD113 with these two antibodies. Cell lines were either completely CD133 positive (namely HCT 116, HT-29A3, LoVo, Colo-206F), negative (LS-174T), or negative with a small subset of positive cells (DLD-1, RKO, SW 480) (**fig. IV. 8 a)**). The minor positive subsets were mainly significant in DLD-1 cell line, in which they occurred repeatedly in following measurements, and represented approximately 17-22 % of population. This was not the case in RKO and SW 480 cell lines, in which positive subsets were less abundant and recognized only by 293C3 clone. Considering the differences between staining with AC133 and 293C3 mAbs neglectable, we further used only AC133 mAb for staining of CD133.

Next we analyzed whether our two different cultivation conditions will have an effect on the cell surface expression of putative CSC markers CD133 and ABCG2. Following 6-day long cultivation in NS-Ac, CD133 levels (quantified by AC133 staining) were higher in one half of the tested cell lines, whereas the second half had none or a slight negative shift in AC133 signal following the their cultivation in NS-Ac medium. ABCG2

was strongly expressed only in LoVo cells, and was reproducibly upregulated under the serum-free cultivation conditions. Low expression of ABCG2 was found also in HT-29A3 and RKO cells and was slightly increased in cell grown in serum-free medium (**fig. IV. 9**).

We were interested whether capability of spheroid formation might be correlated to CD133 phenotype. HCT116 and HT-29A3 are both strongly CD133 positive and form spheres very efficiently, while CD133 negative LS-174T never formed spheres in our hands. We summarized possible correlations in **fig. IV. 8 c)**.

a)



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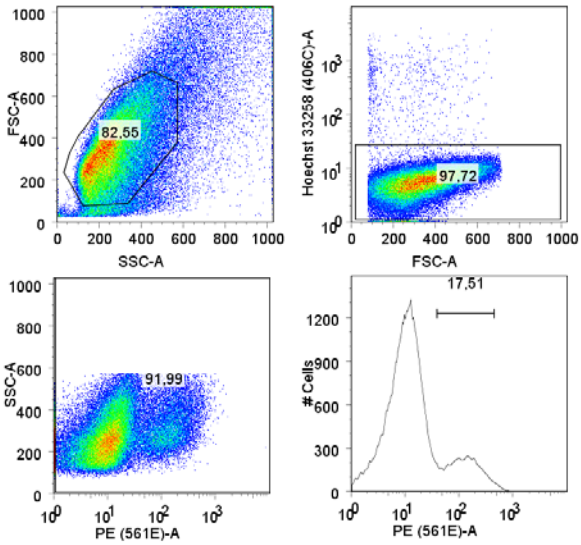


c)

cell line	AC133+	Spheroids
HT-29A3	Y	Y
HCT 116	Y	Y
LoVo	Y	P
Colo-206F	Y	P
DLD-1	P	N
RKO	N	N
SW 480	N	N
LS-174T	N	N

Figure IV. 8: CD133 in colon carcinoma cell lines. a) Flow cytometric analysis of two CD133 epitopes in colon cancer cell lines. Analysis was performed on sub-confluent cells cultured under the standard cultivation conditions and their recommended media for maximum ten days after thawing. Red histogram contours represent respective values of samples, blue histogram contours represent unstained negative controls. **b)** Example of processing raw FACS data (293C3 mAb PE, DLD-1 cell line) **c)** Correlation of AC133+ phenotype with capability of spheroid formation. For better comprehension, values were arbitrarily simplified. (Acronyms: Y - yes, N - no, P - partially).

IV.2.4.2 Effect of serum-free cultivation conditions on cell surface expression of death receptors

To determine whether or not the change in sensitivity to TRAIL-induced apoptosis after differential cultivation was caused by different expression profile of death receptors, we analyzed the expression of DR4, DR5 and Fas and TNFR I by flow cytometry. As

comparison of cells isolated from primary tumors to those from metastases would be valuable, we also extended the set of tested cell lines for CX-1 and SW 620 that are related to HT-29A3 and SW 480 respectively and derived from metastatic syngenic tumors. Primary HT-29A3 and SW 480 share with their metastatic counterparts most of morphological features and are practically unrecognizable in culture. We found analogies also in spheroid forming and proliferation rates.

The expression of death receptors DR4, DR5 and Fas was increased in eight of ten cell lines after cultivation in NS-Ac medium. In our hands, TNFR I wasn't proved strongly expressed on any of ten cell lines. In the two metastasis-derived cell lines (CX-1, SW 620) death receptors were not upreagulated, and Fas profiling showed a minor decrease in CX-1 and RKO (**fig. IV. 9**).

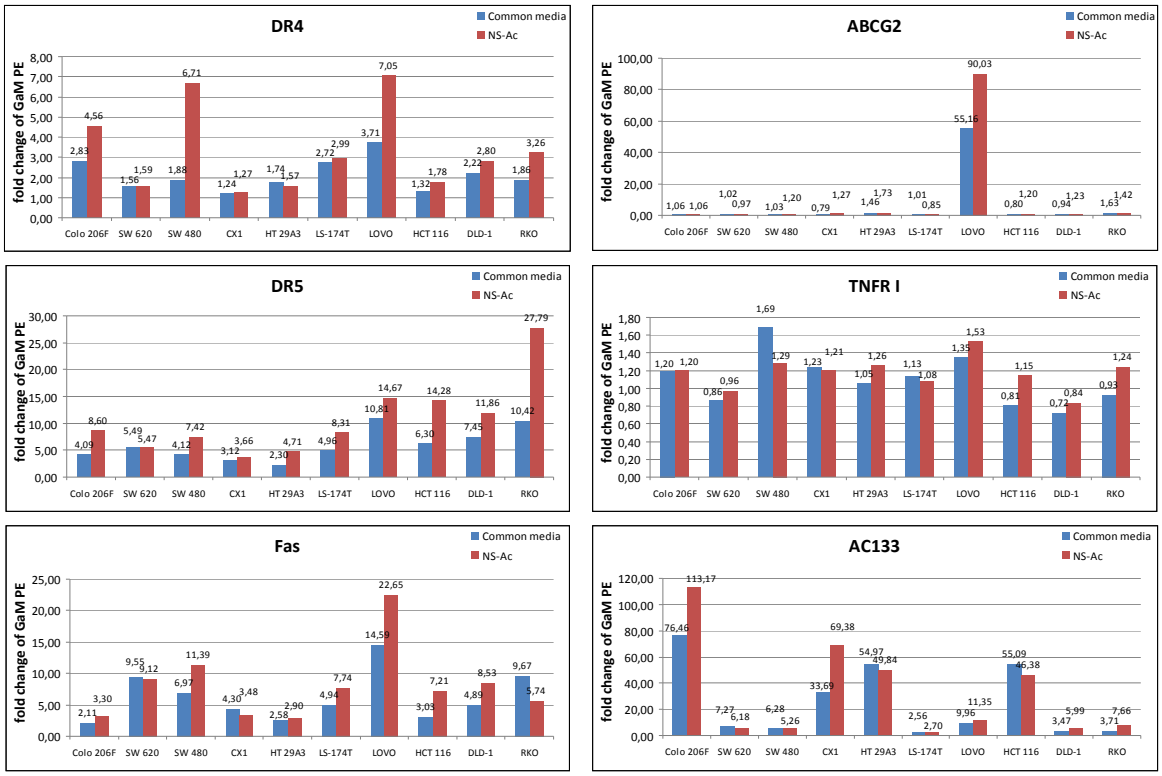


Figure IV. 9: Phenotype of colon cancer cells depending on cultivation conditions. The expression of death receptors DR4, DR5, Fas and TNFR1 and putative CSC markers AC133 and ABCG2 was analyzed by flow cytometry in ten colon cancer cell lines. Cells were cultured in parallel in their recommended media (blue bars) and NS-Ac (red bars) for six days. Cell lines were passaged as necessary when nearing confluence; all cells were sub-confluent at the time of processing. Values of staining negative controls (GaM PE) were used as the reference for plotting the relative expression of analyzed cell surface markers. Experiments were repeated several times with similar results.

IV.3 Influence of fibroblasts on TRAIL-induced apoptosis in colon carcinoma cell lines

The cultivation conditions widely used in the eukaryotic cell biology do not even come slightly close to the situation in organisms, e.g. of cancer cells grown in the tumor environment. It also became recently reported that the neighboring cells e.g. stromal fibroblasts can influence phenotype of other co-cultivating cells and even induce in them changes leading to de-differentiation (Lacina et al., 2007). Thus in the line with this interesting data we were interested whether and how could co-cultivation of fibroblasts (both stromal-derived and normal ones) with colorectal cancer cells influence TRAIL-induced apoptosis the later ones.

IV.3.1 Labeling of cells with fluorescent dyes

IV.3.1.1 Labeling of fibroblasts with CellTracker Red CMTPX

The first important technical aspect of this assay was to distinguish the colon cancer cells from the feeder fibroblasts allow thus their molecular and functional analysis. We were not able to sort out fibroblasts from tested cancer cells on scatter plots; therefore we decided to use red fluorescent dye CellTracker Red CMTPX for labeling of fibroblasts used as feeder cells. Firstly, this would allow us to continue measuring apoptosis by annexinV-FITC, secondly we would minimalize the potential toxicity of the label to the tested cells.

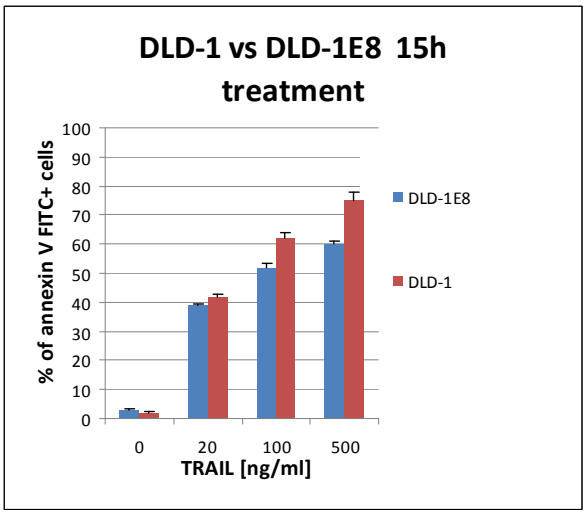
Since we needed a labeling that would remain unchanged for several days we have verified the manufacturer's claims that CMTPX is stable when properly used. However initial testing of mixtures of labeled and plain cells by flow cytometry rendered repeatedly an illegible outcome leaving us with the suspicion that CMTPX staining might be leaky (data not shown).

IV.3.1.2 Preparation and characterization of stable, fluorescent proteins-expressing transfectants of colon cancer cell lines

In another approach that might allow distinguishing feeder and tumour cells we tested labeling the tumor cells with the recombinant lentiviral vector expressing RFP. Although successfully prepared, numerous clones selected from RFP expressing mixed cultures were unstable and ceased to express RFP for yet unknown reasons (data not shown). The experiment design was therefore reduced to single DLD-1 clone E8 that remained positive for RFP expression in long-term culture.

Clone E8 was characterized for its sensitivity to TRAIL in a comparison to the original DLD-1 cells (**fig IV. 10**)**.** Since DLD-1E8 requires permanent selection pressure of puromycin, which would cause bystander killing of fibroblasts during their co-cultivation, we have verified DLD-1E8 reactions to temporary absence of puromycin. In contrast to maternal cell line, DLD-1E8 clone under puromycin selection demonstrated mild resistance to TRAIL, which could be reversed by withdrawal of puromycin. We confirmed visually (data not shown) and later by flow cytometry, that even without puromycin DLD-1E8 cells were able to maintain the required RFP expression for at least one week.

a)



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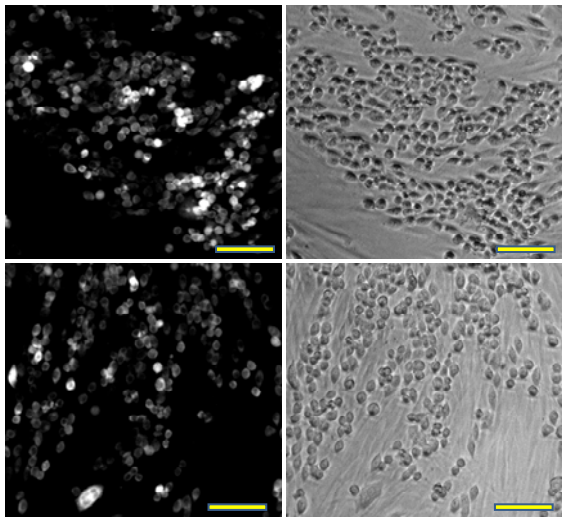

Figure IV. 10: Properties of DLD-1E8 RFP expressing cells. a) Comparison of sensitivity to TRAIL of DLD-1E8 clone and maternal DLD-1 cell line. Bars represent averages of technical duplicates. **b)** DLD-1E8 cells in co-culture with normal human fibroblasts. Cells were not under puromycin selection when photographs were taken due to puromycin-sensitive feeder layer. In both rows, left photograph shows cells in fluorescent spectra; and the right photograph then the same region taken as phase contrast under normal illumination. Yellow bars represent 100μm.

IV.3.2 Fibroblast conditioned media may regulate TRAIL-induced apoptosis

The feeder cells (in our case normal and stromal fibroblasts) might affect the co-cultured cells in several ways. They can soluble factors as cytokines and in-trans change properties of co-cultured cells with expressed receptors for these factors. In addition to the soluble, secreted factors, direct cell-to-cell contact could influence properties and behavior of co-cultured cancer cells.

To determine if fibroblasts produce soluble factors which would influence apoptosis sensitivity of selected colon cancer cell lines DLD-1 and HCT 116, we tested apoptosis of cells cultivated with media conditioned by human fibroblasts and cancerous stromal fibroblasts derived from three different skin tumors (basal cell carcinoma, spinal cell carcinoma and benign fibrous histiocytoma) (**tab. III. 1.**). Experiment was performed in two cultivation time points (1 day (**Figure IV. 11 a)**) and 3 days (**Figure IV. 11 b)**)),

while media were not replaced until adding of TRAIL; 6-hour treatment with TRAIL was used. We observed only minor differences between cells cultivated in conditioned media compared to those in unconditioned controls. A potentially small decrease in apoptosis was found in both DLD-1 and HCT 116 cells after cultivation with conditioned media of S4 and HF. This decrease was more obvious in HCT 116 than DLD-1 cells after 1-day cultivation and faded after 3-day cultivation in both cell lines. Conversely, media conditioned by B3 and B4 seemed to compromise survival of colon cancer cell lines. This effect occurred even in controls untreated with TRAIL, hence was considered unspecific.

a) **b)**

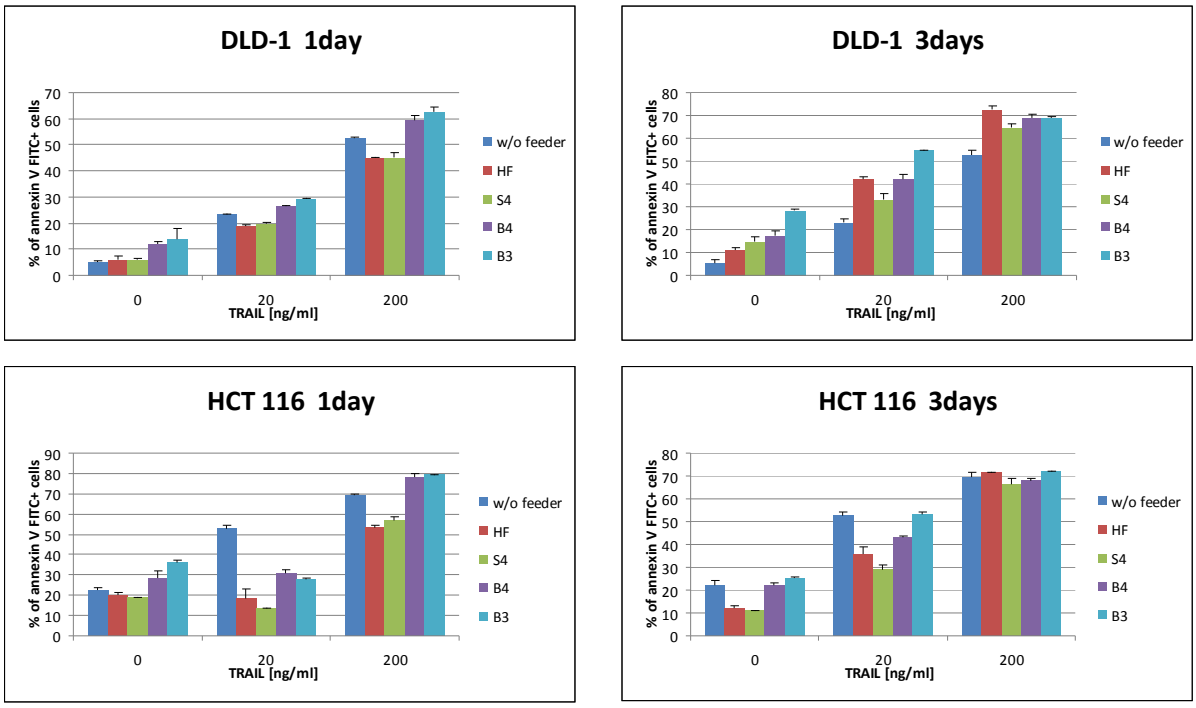

Figure IV. 11: Influence of fibroblasts-conditioned media on TRAIL-induced apoptosis of colon cacer cell lines. **a)** 1-day cultivation in conditioned media from human and stromal fibroblasts. **b)** 3-day cultivation in conditioned media from human and stromal fibroblasts. At both time points, cells were seeded in lower densities to prevent soon reaching of confluence. Media were conditioned for 1 day in cultures of confluent fibroblasts. Before adding to cancer cells, conditioned media were mixed in 1:1 ratio with fresh media recommended for respective cell lines. Bars represent averages of technical duplicates.

IV.3.3 Effect of direct co-cultivation of colon cancer cell lines with fibroblasts on their TRAIL-induced apoptosis.

To determine if direct contact with stromal or normal fibroblasts can regulate TRAIL-induced apoptosis, we performed two types of direct co-cultivation experiments. First, we employed the DLD-1E8 clone expressing RFP enabling distinction of measured cells from feeders on FACS. Cells were cultured together with feeder cells (HF, B4, B3, S4) for 3 days without puromycin.

Flow cytometric analysis with use of annexin V FITC staining revealed no profound protective effect of any feeder type on DLD-1E8 cells. Neither fibroblasts HF and S4 suspected of anti-apoptotic effect in previous experiment demonstrated any clear protection from TRAIL in DLD-1E8 (**fig IV. 12d)**). Since it was reported that puromycin may have influence on apoptosis, and these concerns were supported by our preliminary testing (**fig IV. 12a)**), we measured negative controls without puromycin as well.

In an another approach we analyzed whether co-cultivation of colon cancer cells with fibroblast would in any way affect TRAIL-induced activation of the effector caspases in the Caspase3/7 GLO assay. Both normal and stromal fibroblasts by themselves are resistant to TRAIL-induced apoptosis and did not show any signs of the effector caspases activation (**fig IV. 12b** and data not shown). HCT 116 and DLD-1 cell lines directly co-cultured with complete set of fibroblast types. Cells were seeded on confluent feeders 3 days before adding TRAIL. Cells were incubated with TRAIL in two time scales (2 and 6 hours). Concerning influence of feeders in different incubation times and TRAIL concentrations we have not found any unequivocal trend. When normalized to respective negative controls, activation of the effector caspases was lower in cell co-cultured with S4 fibroblasts in both colon cancer cell lines. Similar effect of B3 feeders was observed in case of DLD-1, while in HCT 116 this was rather restricted to 2-hour treatment (**fig IV. 13**). Thus in these initial experiments we did not observe any significant direct or indirect effect of either normal or cancer-derived, stromal fibroblasts on TRAIL-induced apoptotic signaling in colorectal cancer-derived cell lines. However, at least some tested feeder fibroblasts as spinocellular carcinoma-derived S4 showed at least some (inhibitory) effect on TRAIL-induced apoptotic signaling and also these experiments were done only on

selected colon cancer cell lines. Thus, they need to be repeated on the entire panel of other cell lines and using additional methodical approaches.

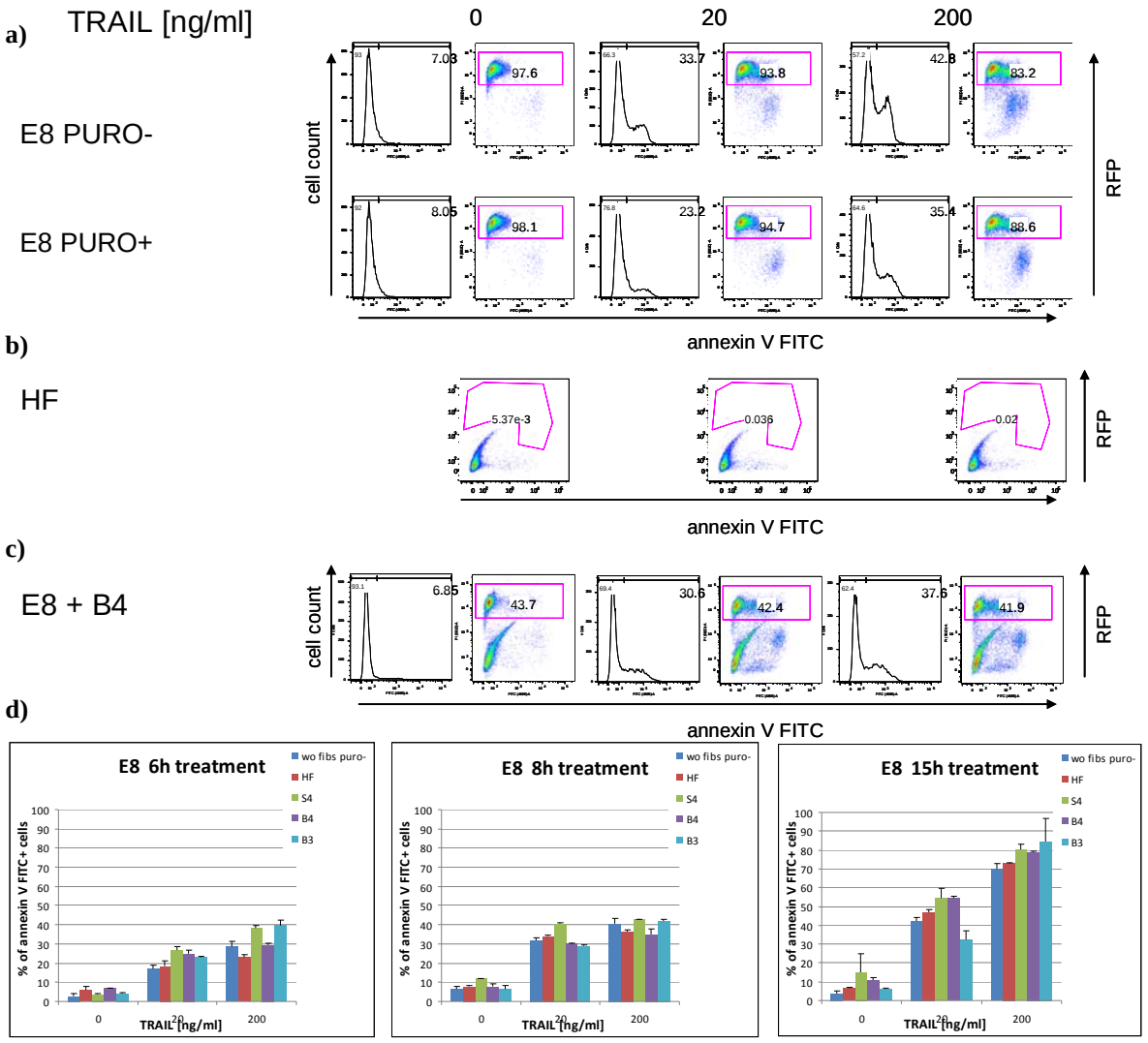

Figure IV. 12: Apoptosis in DLD-1E8 cells after direct co-cultivation with fibroblasts (flow cytometry). **a)** Pure E8 cells were analyzed for TRAIL-sensitivity depending on selection agent puromycin. Cells were either kept under puromycin (3 µg/ml) selection during cultivation and treatment with TRAIL - until processing for Annexin V FITC staining; (entitled E8 PURO+) or puromycin was withdrawn from the cultivatiom medium 2 days before treatment of cells with TRAIL; (entitled E8 PURO-). **b)** Verification of resistance of feeder fibroblast cells to TRAIL by Annexin V FITC on FACS. TRAIL (concentrations 0; 20; 200 ng/ml) was used for 15-hour treatment. Similar results were obtained with all fibroblasts used (B4, B3, S4, HF). Results from annexin V FITC staining were further confirmed by M30 FITC assay (not shown). **c)** TRAIL-induced apoptosis in direct co-culture of E8 cells with B4 fibroblasts used as feeders; (example of processing FACS data). **d)** E8 cells cultured with/without confluent fibroblasts for 3 days were treated with TRAIL in timescale from 6 to 15 hours and analyzed by flow cytometry on annexin V FITC staining. Bars represent averages of technical duplicates.

a) **b)**

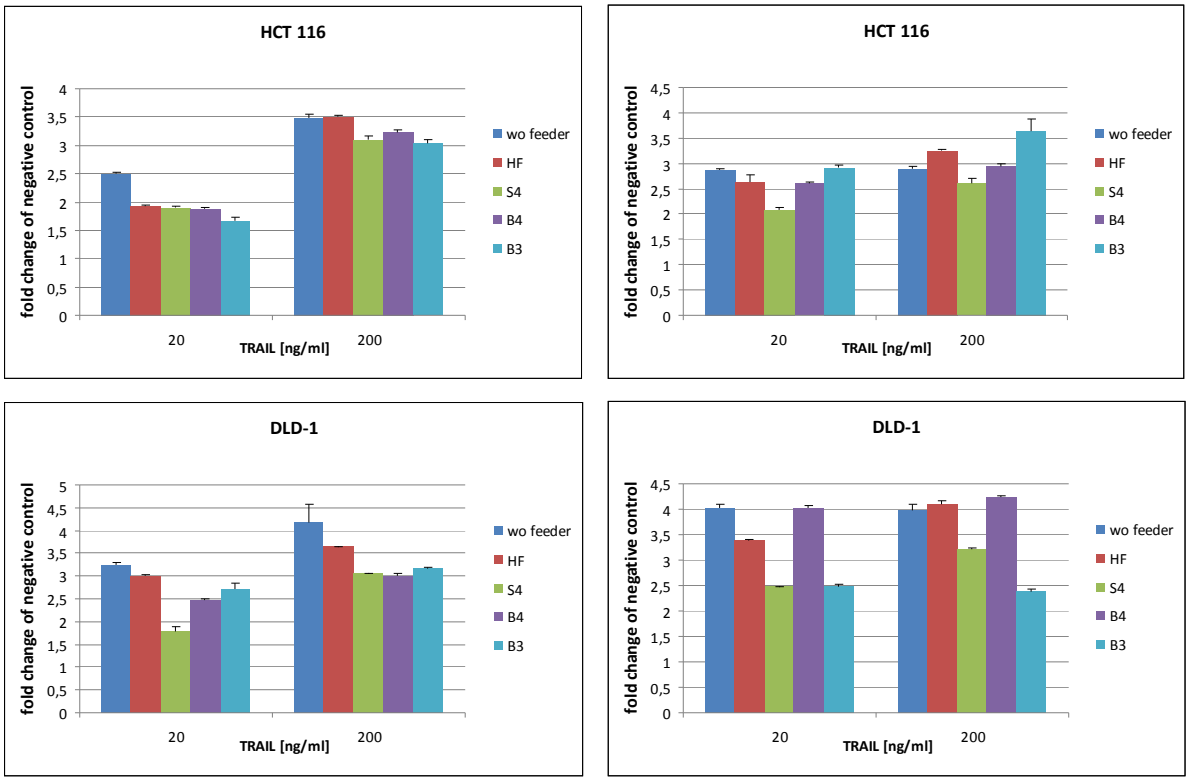

Figure IV. 13: TRAIL-induced apoptosis of HCT 116 and DLD-1 cells after co-cultivation with stromal fibroblasts (caspase 3/7 GLO assay). HCT 116 and DLD-1 cells were co-cultured with normal human fibroblasts (HF), stromal fibroblasts from spinocellular carcinoma (S4), basalioma (B3) and benign fibrous histiocytoma (B4). Cells were seeded on plates with confluent fibroblasts and plain plastic as a negative control 3 days **b)** before treatment with TRAIL. TRAIL was added diluted in fresh media in concentrations 20 and 200 ng/ml. Cells were processed after **a)** 2-hour **b)** 6-hour treatment. Bars represent average RLU values of technical duplicates normalized to RLU values of respective untreated controls.

V. Discussion

When thinking of tumorigenesis, several seemingly easy but fundamental questions have not been answered so far. Especially, our apprehension of the very first step of tumor initiation is based mainly on indirect data and only recently we became aware of the importance of microtumor environment for the initiation and progression of tumorigenesis. Therefore any novel consequences may ultimately lead to complete shift of current paradigm. Nowadays, there is no question that cancer is a genetic disease that starts from a single cell. However, at least in carcinomas, it is more and more likely that not mutations per se but rather combination of acquired genetic and epigenetic changes within the cells and their interaction with surrounding cells and also avoidance of the immune response actually must cooperate for the successful tumor initiation (Baylin and Herman, 2000; van Zijl et al., 2009). Extrapolating the recently reborn cancer stem cells (CSCs) hypothesis in this manner may generate verifiable predictions which we took into account when designing our experiments.

CSC hypothesis implies that cancer cells are heterogeneous in their self-renewal capacity and, contrary to what clonal evolution theory assumes, this is associated with hierarchy. Together, this predicts a pivotal population of CSCs and heritability of its exclusive traits. The central question of our work was, whether we would be able to (i) observe such heterogeneity in cancer cell lines, and eventually, (ii) examine their sensitivity/resistance to TRAIL-induced apoptosis. Many would object that cell lines are not suitable model for studying CSCs as cultivation of cells for prolonged period leads to expansion of fast proliferating possibly non-CSC clones, and cancer cells with SC-like properties may therefore vanish from such cultures after a number of passages. Although these concerns seem well-founded and fresh tumors remain the primary source of CSCs for their characterization, our current insight into social context of cancer cells is too limited and many groups have obviously isolated the CSC entity from established cell lines (Dou et al., 2007; Charafe-Jauffret et al., 2009). Moreover, utilization of cancer cell lines may be advantageous, since cell lines unlike tumors do not have any contaminating normal stem cells that quickly differentiate under the normal culture conditions (Kondo, 2007).

We have analyzed eight colon carcinoma cell lines for their morphological and biochemical changes imposed by the spheroid assay (SA) cultivation conditions. SA was originally developed for characterization of normal tissue stem cells and has recently been used for enrichment of putative CSCs from solid tumors in many studies (Galli et al., 2004; Grimshaw et al., 2008; Inagaki et al., 2007; Ricci-Vitiani et al., 2007). In general, we observed morphology of most tested cell lines was seriously influenced and notably some cell lines including HCT 116 and HT-29A3 demonstrated capability of forming spheres or spheres-like structures. We haven't verified the tumor initiating capacity of sphere-forming cell lines *in-vivo*; still our results suggest at least promising morphological analogies with stem cells exist in some cell lines. One thing that must be taken into consideration when interpreting the spheroid assay is that there is little definitive information as to which cells are being propagated. For example, in neurosphere assay, so far best-studied sphere model, it has been shown that neurosphere frequency approximates progenitor cell activity more closely than stem cell activity (Marshall et al., 2007). Moreover, after dissociation of neurospheres and their subsequent re-plating more than 90% of cells die which suggests that spheres are also implicitly heterogeneous (Reynolds and Rietze, 2005). Thus we were aware of this fact and considered spheres as the changed environment for the selected cell lines due to their 3-D structure more resembling actual original tumor and not as a source of completely more progenitor-like cancer cells. Another related puzzling issue about spheroids is the very mechanism of their emergence and maintenance. Undisputed is the effect of serum withdrawal that makes cell aggregation easier and this effect seems rather nonspecific. Nevertheless, creation of the round-shaped aggregates is presumably accompanied by cytoskeleton remodeling and one may expect that also specific intercellular junctions and signaling are being active within the spheres. Finally, the pivotal distinguishing feature must be undoubtedly overcoming the anoikis. It has been suggested that only cells responsive to EGF and bFGF are supported for survival, whereas the unresponsive differentiated cells die early on mainly via induction of apoptosis (Reynolds and Rietze, 2005). However, we have not observed any excessive dying of cells of any cell line upon seeding in NS-Ac medium; moreover we have shown that EGF and bFGF are not strictly required for the formation of spheroids but for their maintanace and might also limit number of cells within these spheroids. Based on these observations we speculate that: (i)

cells of cancer cell lines may get along the restrictive conditions despite not all being *bona-fide* cancer stem cells. Providing that cancer cell lines were derived from single cancer cells/cancer stem cells (if CSC theory would be applied) they may all posses at least some degree of stem-like properties. Alternatively, transformed phenotype and adjustment to artificial in-vitro conditions provide sufficient fitness for survival even in serum-free medium, (ii) slower proliferation of cells within spheroids could be a sum of a number of influencing factors including denial of some serum-based ones (Satoh et al., 1996), less efficient accessibility of cells within spheres to nutrients or by non-optimal composition of the NS-Ac media (/proliferation supplement).

In order to prove "stemness" and namely self-renewal, not actually primary but secondary, tertiary and higher orders sphere formation is required. Indeed, it is of interest to investigate why at all CSCs as well as normal stem cells are enriched in the spheres. It has been reported previously, using sphere assay, that that Hedgehog-Gli signaling is implicated in regulating the self-renewal and tumorigenesis of CD133+ CSCs in gliomas (Clement et al., 2007). In one recent study EGF receptor signaling has been shown to synergize with Hedgehog (hh)/Gli in oncogenic transformation (Schnidar et al., 2009). Taken together, EGF-Hedgehog self-renewal axis might serve as a connection link between CSC and their maintenance in spheroid cultivation. Our experiments with omitted EGF were too short to explore possible terminating effect on self-renewal; in fact we have never passaged the spheres more than 3 times for purely pragmatic reasons. We also cannot exclude a possibility that the examined colon cancer cell lines produce by themselves low but sufficient amount of the necessary growth factors including EGF that would allow for their survival and limited proliferation. We will surely address this topic in our future experiments.

We have investigated the surface expression of death receptors and stem cell markers CD133 and ABCG2, how it changes in relation to differential cultivation, and eventually if this influences the TRAIL-induced apoptosis. Surprisingly, CD133 (CD133-1 epitope) was strongly expressed by all cells of some cell lines (HCT 116, HT-29A3, LoVo) while it was completely absent in others (RKO). This is in contrast to results usually obtained in fresh tumor samples, where stem cell marker expression tends to be much more heterogeneous, and marker-positive subsets are often minor (Ricci-Vitiani et al., 2007). Our

results suggest that cell lines derived of one cancer cell maintain its original marker expression profile, perhaps even related hierarchical status. However, to claim that some cancer cell lines more or less represent clones of CSCs, while other do not, would be very controversial and a clear over-interpretation at this point. Even so we could delineate some correlation between AC133+ phenotype and efficacy of spheroid formation as all spheres were formed in AC133+ cell lines (HCT 116, HT 29A3) and conversely, none of AC133- cell line (DLD-1, RKO, LS-174T) formed spheres in our hands. However, not all AC133+ cell lines (LoVo, Colo 206F) formed spheres, although some rare cases were noted in prolonged cultivations. In addition to this, DLD-1 cell line has perhaps the most interesting phenotype as it maintains almost stable subset of AC133+ cells wobbling from 17 % to 25 % of all cells, which was verified repeatedly by flow cytometry. It has been recently described in literature, that DLD-1 cell line retains CSC-subpopulation, which is i.a. characterized by high expression of Nanog, CD44 and downregulation of CK20 (Ferrand et al., 2009). These findings may oppose our previous suspicion: not only may the cancer cell line represent uniform hierarchal caste but it may further develop its own internal hierarchy. Besides recognizing CD133 phenotype as a potential determinant of cell morphology during serum-free cultivation, we found that NS-Ac alone influenced the surface expression of CD133, or precisely its AC133 epitope. Among the AC133+ cell lines, Colo 206F and CX-1 showed remarkable increase in AC133 expression (+48 % and +106 % respectively), while only minor increase was observed in LoVo and mild decrease in HCT 116 and HT-29A3 cell lines. This result either indicates that enrichment in stem cell-like entity during serum-free cultivation is not obligate, or using of AC133 as a marker is misleading. AC133 was also relatively markedly upregulated in RKO and DLD-1 cell lines (+106 % and +74 % respectively). In DLD-1 increase was marginal with unchanged proportion of AC133+ subset. Mainly in case of AC133- cell line RKO, biological significance of AC133 epitope upregulation is umbrageous, because the absolute change seems neglectable. All in all we walk into question, as to what is the relation between AC133, differentiation and spheres. At present, lack of information on physiological function of CD133/prominin-1 together with fact that we performed only one set of experiments complicates the proper interpretation of our results. At least promising indicia emerged from a recent study by Qiang et al., (2009), that prominin-1 is involved in

cytoskeleton alteration and function of glycogen synthase kinase 3 (GSK3), pivotal protein in self-renewal regulation.. We will undoubtedly modify our experimental setup in order to gain more reliable statistical data. In addition, performing inverse experiment, based on returning the cells into their regular growth media after cultivation in NS-Ac will address the findings of Vodianik (2005) and others that differentiation decreases the level of CD133 protein.

Strong surface expression of another often used CSC marker ABCG2 transporter was detected only in LoVo cell line and it was even increased after cultivation of LoVo cells in NS-Ac. Other cell lines demonstrated only low (HT 29A3, RKO) or none expression of ABCG2. Our observation is in accordance with previous studies in which EGF was described to significantly upregulate ABCG2 expression in cytotrophoblasts, BeWo and MCF-7 cells (Meyer zu Schwabedissen et al., 2006).

As TRAIL-induced apoptosis appears to be closely connected to the regulation/protection from tumorigenesis, at least at the metastatic progression, and this system is also recently being explored for cancer therapy, the obvious question for us was if and how the cultivation conditions affect TRAIL-induced apoptotic signaling in analyzed colon cancer cell lines. Several studies suggested TRAIL as a potential component of immune surveillance, and especially, of anti-metastatic mechanisms (Cretney et al., 2002; Grosse-Wilde et al., 2008). Moreover, an elegant study of Mani has recently linked epithelial-mesenchymal transition (EMT) to acquisition of cells of stem cell properties (Mani et al., 2008). Whereas EMT is regarded as crucial step enabling cancer to metastasize, it might also play a role in the initial phases of tumorigenesis, so we anticipated that TRAIL might consequently play role in early stages of tumorigenesis by killing cancer stem-like cells. In addition, some clues supportive of this assumption have already occurred in literature (Sussman et al., 2007).

Flow cytometric analyses performed on ten colon cancer cell lines from normal cultivation versus serum-free cultivation, pointed out generally elevated levels of surface TRAIL-R1 (DR4), TRAIL-R2 (DR5) and Fas (CD95) in most cell lines grown under the serum-free conditions, while outcomes of TNFR1 were considered rather neglectable. After serum-free cultivation, DR5 was most upregulated in RKO cells, while DR4 as well as Fas mainly in SW 480 and LoVo cell lines. Instead of complete listing of changes in individual

cell lines, let's concentrate on important patterns and eventual deduction of their origins. First of all, the fact that in several cell lines at least some death receptors are not upregulated, suggests that the effect of serum-free conditions is not unspecific (e.g. metabolic). For instance, Fas was nearly 41 % down after cultivation without serum in RKO cells. As far as the upregulation of DR5 is concerned, it has been described in literature that NF-kB may be involved. Interestingly, according to one recent study, NF-kB activation discriminates the character of signals in order to either upregulate DR5 or not (Shetty et al., 2005). While both etoposide and EGF activated NF-kB pathway, only etoposide was shown to increase the level of DR5, since unlike of EGF, etoposide did not recruit histone deacetylasse 1 (HDAC1) to the DR5 gene. This is fuelling our speculations, that EGF dependant NF-kB activation might (purely hypothetically) also be the cause of DR5 shift, provided that HDAC1 was for any reason inactivated. We suggest that we should perform extended experiments, either by withdrawing EGF from NS-Ac or simultaneous measuring of HDAC1 activity prior to measuring the death receptors. Particularly for measuring the effect of serum-free conditions on the phenotype of cancer cell lines, we widened the set of cell lines for CX-1 and SW 620, metastatic relatives of HT-29A3 and SW 480 respectively. We have observed a striking similarity in behavior of CX-1 and SW 620 in comparison to their primary tumor-derived counterparts, and that was almost untouched expression profile of all tested markers after serum-free cultivation. It is a tempting idea that this is due to a selection of cells during the metastasizing, which prefers cells adjusted to overcoming anoikis and restrictive conditions. These cells therefore do not need to undergo further phenotype changes to get along with NS-Ac. In support of such concept is a recent finding of Laguinge et al. (2008) that DR5 mediates anoikis in human colorectal carcinoma cell lines.

Upregulation of death receptors during the SA is what one would anticipate when assessing TRAIL as a selective anti-CSC agent. However, our data obtained from measuring the TRAIL-induced apoptosis were rather contradictory. To sum up, we have employed several principal approaches for the assessment of TRAIL-induced apoptosis, including two based on flow cytometry (M30 FITC, annexin V FITC staining) and also different time scales all confirming that in most colon cancer cell lines (except for LoVo and Colo-206F) their cultivation in the serum-free NS-Ac medium compromised the

TRAIL-induced apoptosis. We have not found any reliable correlation between spheres and TRAIL resistance. Assigning the resistance to simple explanation that TRAIL cannot penetrate the colonospheres is therefore infirmed. In a recent similar study done on lung cancer A549 cell line, Yang (2009) not only neither assigned the resistance of spheroids to a limited diffusion of TRAIL, but suggested upregulation of anti-apoptotic Bcl-2 as a responsible mechanism. Another reason for the acquired resistance of spheroids to TRAIL-induced apoptosis might be upregulation of the caspase-8 competetive inhibitor FLIP, as was recently documented in CD133-positive population of the breast carcinoma MCF-7 (Zobalova et al., 2008). Further we must admit the possibility that signaling from EGFR alone may cause the resistance, since it was many times reported to have various profound anti-apoptotic effects (Akca et al., 2003). For example, in one such study EGF-mediated Mcl-1 upregulation was reported to block TRAIL-induced apoptosis (Henson et al., 2003). On the other hand, both signaling pathways from death receptors and EGFR appear to be very complex as documented in reports of EGF-induced apoptosis (Grudinkin et al., 2007; Zhao et al., 2006) or TNF induced survival mediated by EGFR (Yamaoka et al., 2008). Although multiple unknown regulatory knots remain to be solved in upcoming experiments, at this point we conclude that 3D spheroid model may be a better model for the multicellular resistance found in solid tumors than cells grown as monolayer.

By observing that AC133 positive cell lines may be both highly (HCT 116) or less (HT-29A3) sensitive to TRAIL-induced apoptosis and *vice-versa*, we conclude that no simple correlation between the stem cell marker AC133 and TRAIL sensitivity was found, at least in our model colorectal carcinoma cell lines.

In an attempt to even more mimicate the real tumor conditions, we assessed TRAIL-induced apoptosis of selected cancer cell lines co-cultivated with fibroblasts isolated from tumor stroma. The logical consequence with previous CSC context is retained, since Smetana's group has recently shown that certain stromal fibroblasts may induce dedifferentiation in co-cultivated keratinocytes (Lacina et al., 2007). We have imitated their co-cultivation approaches and moreover developed our own direct co-cultivation system with use of RFP-labeled cancer cell line. At first, we observed that medium conditioned by normal fibroblasts (HF) and even more by fibroblasts from spinocellular carcinoma (S4) possibly inhibited TRAIL-induced apoptosis in HCT 116 and

DLD-1 cells. Such effect occurred mostly at TRAIL concentrations as low as 20ng/ml and was lost after 3 days, when conditioned medium was not exchanged. This may witness for a soluble factor that is either unstable or exhausted after three days. Interestingly, similar effect we observed in direct co-cultivation of the same cancer cell lines, at least with S4 fibroblasts. The fact that only at low concentration of TRAIL the effects were demonstrated seems logical. Indeed, the higher TRAIL concentration used (200 ng/ml) is artificial, and unlikely to ever occur in organism. Conversely, we have observed mild increases of cell death in TRAIL-untreated controls. Mainly after co-cultivation with B3 fibroblasts from basal cell carcinoma effects seemed ambiguous depending on whether direct co-cultivation or conditioned medium was used. Moreover, no influence of the feeder fibroblasts on TRAIL-induced apoptosis was reliably recognized in RFP-expressing DLD-1E8. In this particular case, however, the possibility that E8 clone has unique response, different of maternal cell line cannot be definitely excluded. It must be noted that all observed effects were minor and potentially bordering with statistical deviation, and we shall continue experiments to reliably address this potentially very interesting and important problems. Importantly, most of co-cultivation reports so far published was done in heterologous animal models, and even our setup is quite artificial. In future, we will therefore try to obtain stromal fibroblasts from colorectal carcinomas, as well as use the current fibroblasts in combination with cancer cell lines of different origin (e.g., breast, prostate and head and neck carcinoma). This approach should answer the question, if eventual influence of fibroblasts is panspecific or specific to tumor of origin. In conclusion, we have a clear motivation to continue in experiments since we believe in immense potential of co-cultivation studies, so far in their infancy. We have already tuned the co-culture protocols and set-up tools necessary for sophisticated flow cytomeric analyses. The fact that our current data are rather indistinctive does not discourage our efforts and even stimulates us to develop better experimental design utilizing experience from the so-far obtained results.

Conclusions:

1. We have succesfully characterized a set of colon cancer cell lines and changes of their morphology, sensitivity to TRAIL-induced apoptosis, and expression of selected surface markers depending on cultivation conditions.

2. We have observed that colon cancer cell lines were mostly uniform in their properties *in vitro* in relation to cancer stem cell model. Most of them posses uniform expression of putative CSC marker AC133, except for DLD-1 cell line with stable minor subset of AC133 positive cells. We have further observed correlation of AC133 phenotype and stem cell-like properties (sphere forming, apoptosis resistance).

3. We have developed experimental design for direct co-cultivation of colon cancer cells with fibroblasts, (eventually with various cells of tumor stroma).

4. We have shown that most of tested colon cancer cell lines were to some extent sensitive to apoptosis induced by TRAIL. Moreover, TRAIL agonistic receptors were upregulated after cultivation of cells in CSC-prone conditions. Paradoxically, apoptosis was mostly compromised despite the upregulation of death receptors.

VI. Epilogue

Respecting the fact that any game with the Occam's razor is usually dangerous, author somehow doesn't dislike being called heretic. Flipping through the results and myriads of literature on the topic, a repeated urging idea comes to his mind that something is overlooked in our very basic view of tumorigenesis. Irrespectively of whether clonal evolution or CSC theory or some kind of their mutual integration is favorized, we always assign the cancer causality to mutations. This belief, like most dogmas, is founded on lack of experimental evidence to the contrary. Author dares to claim, that regardless the well founded role of mutations, viewing cells in their social context is still much neglected.

Hence the role of cell communication in regulation of self-renewal symmetry, eventual induction of stem-cell properties and novel concepts of tumorigenesis represent the scaffold of author's highest research priorities in possible future research career.

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